

Targeting Cancer-Specific Mutations with RNA-Triggered Chromatin Shredding

Corresponding Author: Professor Jennifer Doudna

Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

In this manuscript titled "Selective Elimination of TP53 Mutant Cells by Transcript-Activated Chromatin Shredding", the authors present in vitro and in vivo data on the selective elimination of cells containing transcripts that match the guide RNA in a recently described CRISPR system, Cas12a2. Cas12a2 detects target RNA and degrades both the target RNA and trans double-stranded DNA without sequence preference. The data presented are consistent (though not fully supportive) with a model in which Cas12a2 can be used to deplete cells harboring mutant genes.

Although the authors provide several examples, including GFP targets and various oncogenes, the primary focus is on TP53, where mutations may induce tumor formation but the mutant protein itself cannot be directly targeted with small-molecule drugs. Instead, the proposed strategy is to target the transcript produced from the mutant gene. The most intriguing candidates are single-point mutations that presumably maintain normal p53 protein levels but nevertheless promote tumor initiation and growth.

The in vivo examples are potentially exciting, particularly if they can indeed target lung tumors using a recently developed LNP formulation. However, I have major technical concerns and cannot recommend publication until these issues are addressed with additional experiments. My concerns fall into two major categories:

- (1) Lack of adequate controls to support the claim that cell elimination is based on transcript sequence rather than transcript abundance in most experiments.
- (2) Low statistical power of the in vivo data.

Specific Comments

1. While I appreciate the authors' transparency regarding the need to treat mice six times with LNP deliveries over several days due to low targeting efficiency (only ~10% of tumor cells receive them), they should provide data confirming that the mouse lung and other physiological parameters remain normal after repeated treatments, as LNPs may accumulate elsewhere.
2. Figure 5d (in vivo data): More data points are needed to support any strong claim of selective tumor cell elimination. The difference between PBS and LNP-MYC appears marginal, with only a single significance marker (unclear which statistical test was used and how many animals were included). This is not convincing. Even more striking is that the difference between LNP-NT and LNP-MYC is even smaller, and no statistical measure is provided. These groups must differ for the authors to claim selectivity. Additional animals are needed before making such claims.
3. Continuing with Figure 5d: The authors should perform experiments targeting tumors that do not carry the targeted mutation to demonstrate selective elimination of TP53 mutant cells. While they show some cultured cell data in Figure 4, the absence of such in vivo controls makes it difficult to accept their claim of selectivity.
4. Figure 5d: The manuscript does not describe what the NT gRNA represents.
5. Figure 5f: The only time point showing a significant difference between PBS and LNP-p53 R248Q is at 15 days. Other time points (11 and 22 days) show no difference. More animals are needed before making definitive claims about selectivity.
6. Continuing with Figure 5f: Again, the NT guides are not described. Additionally, the authors should include data from tumors lacking the targeted p53 mutation before claiming selective in vivo tumor reduction via the proposed mechanism.
7. Overall, the in vivo data require additional evidence to support the proposed mode of action.
8. Extended Data Figure 1: 550 transcripts per cell is a relatively high transcription level, yet the authors observed only

modest growth defects. This suggests the approach may require unusually high RNA expression. If this is a function of RNP concentration, the authors should characterize the transcript copy number required for cell killing at higher RNP concentrations. The statement "Control cells with no GFP expression were unaffected" may hold at low RNP concentrations, but what about higher concentrations where fewer target transcripts might suffice?

9. Extended Data Figure 1f: The authors state that DNA cleavage in trans occurred at 0.1 mM Mg^{2+} , but the gel shows that most trans DNA remains uncleaved even after 1 hour. The data do not align with the claim. Quantification is needed (e.g., "2% of DNA was cleaved"), and replicates should be included to confirm reproducibility.

10. Figures 3b–e: The comparison between mutant and wild-type transcripts was made using two different cell types (RPE1 vs. PC9), which is not convincing. The authors did not compare transcript levels between these cell types. Given that GFP targeting shows dependence on transcription levels, they must demonstrate that transcript levels are comparable here.

11. Figure 4e: The competitive growth comparison between mutant and wild type does not account for transcript abundance differences. Extended Data Figure 4f suggests wild-type cells have 20-fold fewer transcripts. If true, the enrichment of wild type in competitive growth assays cannot be attributed to mutant-selective cell elimination.

12. When comparing R175H as a control, the authors observe similar transcript counts as R248Q yet see a growth phenotype only for the targeted mutation. This appears to be the only experiment where mutant and wild type are compared side by side. Why did they not perform a competition assay, which could be easily done via amplicon sequencing?

13. Extended Data Figure 5a: The authors claim that "trans DNA cleavage by Cas12a2 RNPs containing R248Q gRNA was specific to the R248Q mutant transcript," but the figure shows that most trans DNA remain uncleaved, indicating low Cas12a2 activity. Replicates and quantification are needed.

14. The statement "These results indicate that Cas12a2 RNPs can precisely target TP53 mutant cells with high selectivity and minimal side effects in healthy cells" is problematic. Mutant cells appear healthy when treated, so calling controls "healthy" is misleading. More importantly, the authors must show that wild-type cells with equivalent transcript abundance are not eliminated. This is critical because they claim, "Taken together, these results demonstrate that SNVs spanning TP53 mutant transcripts can be exploited for Cas12a2-mediated selective cell killing." Selectivity must be demonstrated convincingly.

15. The sentence "Cas12a2 showed trans-cleavage selectivity in vitro with two of the gRNAs (gRNAs 4 and 6) targeting the M246I mutant transcript, suggesting a single mismatch between the gRNA-target duplex can decrease Cas12a2 activity" is unclear. Why does this observation suggest that a single mismatch reduces activity?

16. "Cas12a2 targeting with one of the selective gRNAs, M246I gRNA 6, induced growth defects in NCI-H23 cells (p53 M246I) but not in U2OS cells (p53 WT)." I have similar concerns here. The authors are comparing different cell types, which does not rule out the possibility that the observed difference arises from cell-type-specific factors or differences in transcript abundance.

17. It would be helpful for the authors to explain why they target the mutation through the PFS rather than through the protospacer.

18. Non-target guides: I could not find information about what was used as non-target guides. There are many experiments with different targets in this manuscript. Did the authors use the same non-target guide for all experiments, and if so, why was it chosen?

19. Figure 3: This figure as a whole is problematic because the authors did not control for RNA levels. Unless they can demonstrate that transcript levels are similar between PC9 mutant and RPE1 wild type, I suggest removing this figure. Without such evidence, they cannot claim that cell elimination is selective for the targeted mutation.

Summary:

This is a potentially important manuscript of broad interest; however, there are numerous major concerns that must be addressed before publication.

Referee #2

(Remarks to the Author)

Zeng et al. report an innovative strategy for selective cancer cell killing by harnessing Cas12a2, an RNA-activatable nuclease that cleave trans-DNA non-specifically upon sensing cognate RNA transcripts. Building on two earlier studies by others that characterized Cas12a2's RNA-activated, trans-nucleolytic biochemical activities, this work provides a logical and timely extension of Cas12a2's usage into mammalian cells. The authors first show that once activated by a target reporter and endogenous transcript, SuCas12a2 triggers acute DNA damage and cell-cycle arrest in cultured human cells. By exploiting signature mutations in cancer-associated transcripts, including an EGFR in-frame deletion and the most common p53 SNVs, they further demonstrate that Cas12a2 can discriminate mutant from WT transcripts with selectivity, leading to potent killing or proliferation arrest across multiple cancer cell lines. Finally, and impressively, LNP-mediated delivery of Cas12a2 targeting the p53 R248Q mutation enables in vivo tumor burden reduction in MYC-driven liver cancer and lung cancer xenograft models.

Overall, this study is conceptually bold, novel, technically sound, and presented with clarity. The central intellectual advance, selective cancer cell killing, is arguably the most appropriate and compelling application of Cas12a2 in eukaryotic settings. Importantly, the work helps address a major unmet medical need in oncology, namely the long-standing difficulty of targeting undruggable oncogenic signatures such as TP53 mutations. Given the strength of the findings, their clinical relevance, and the potential — with continued development — to establish a new paradigm in programmable and precision cancer therapeutics, this study will be of broad interest to the research community.

Below I outline several substantive concerns and recommendations, including one major issue (the lack of a systematic single-nucleotide mismatch discrimination analysis, see below for details) that must be addressed to ensure the platform's

rigor and generalizability.

1. To establish Cas12a2 as a generalizable cancer killing platform, the manuscript would benefit from a more systematic investigation of 1-nt mismatch tolerance in human cells. While the p53 R248Q targeting data in Fig. 4, 5, and S6a are compelling—demonstrating that placing the oncogenic mutation within the PFS region confer strong selectivity—it is unclear what fraction of oncogenic mutations can realistically benefit from this A-rich PFS discrimination strategy. It is likely only a modest subset. As shown in Fig. S6i, 73% of TP53 mutations have nearby adenines that could serve as PFS motifs, meaning that in most cases the pathogenic nucleotide would reside within the guide region rather than within the PFS itself. For these more common scenarios, defining Cas12a2's single-nucleotide mismatch discrimination rules within the guide region is essential, particularly because the original 2023 bacterial study reported promiscuous mismatch tolerance. This underscores the need to rigorously characterize mismatch discrimination in human cells, where therapeutic applications demand much higher precision. Such insights would also inform users in designing effective gRNAs.

I suggest that the authors conduct a more systematic analysis in which one or more TP53 loci are tested with 1-nt mismatches introduced at multiple positions across the protospacer. Such data would provide crucial insights into targeting specificity and significantly strengthen the platform's generalizability.

2. Along the same line, the description of the PFS requirement for SuCas12a2 could be clarified to better orient the readership. In the current manuscript, it is only briefly mentioned on p.9 in reference to Fig. 4, "SuCas12a2 recognizes an adenine-rich PFS site adjacent to a targeted RNA sequence". However, the original study that defined the PFS (PMID: 36599979; 5'-GAAAG 3' PFS) provides a more precise specification of its length and nucleotide identities. Introducing this PFS information more explicitly and much earlier in the manuscript (i.e., before any RNA-targeting experimental results are described) would help readers better understand the basis for the RNA-targeting designs that follow.

3. Also related to targeting selectivity, the following data interpretation for Fig. S6f (p.11) is a bit misleading: "Cas12a2 showed trans-cleavage selectivity in vitro with two of the gRNAs (gRNAs 4 and 6) targeting the M246I mutant transcript, suggesting a single mismatch between the gRNA-target duplex can decrease Cas12a2 activity (Extended Data Fig. 6f)". However, this conclusion appears inconsistent with data shown in Fig. 6f, where genomic DNA shredding by Cas12a2 is measured in the presence of WT versus mutant p53 target transcripts. g2, g3, and g5 induced similarly minimal or no degradation for both targets. g1 shows modest degradation for the mutant but not the WT transcript. Most importantly, g4 triggered strong degradation for both targets—although cleavage was somewhat higher for the mutant, the WT transcript still induced substantial degradation. Thus, describing g4 as "selective" based on the slightly decreased activity is technically not wrong, but quite misleading. Overall, g6 is the only guide that demonstrates clear and comfortable selectivity. The interpretation of Fig. S6f should be revised accordingly to more accurately reflect the data.

Again, only one out of six guides demonstrated meaningful selectivity, underscoring the critical need for a comprehensive characterization of Cas12a2's 1-nt mismatches discrimination across multiple targets using in human cells a full panel of mismatched guides. This is a major concern that shall be dealt with prior to publication.

4. For clarity, it would be helpful if the figure legends specified whether the error bars represent SD or SEM. Please adding this information consistently across all figures to improve readability.

5. The statistical analysis for the LNP-MYC sample in Fig. 5d should be performed relative to the NT control, as is done consistently across all other panels, rather than relative to PBS. Using PBS here may confound interpretation, as NT is the proper statistical inference.

Referee #3

(Remarks to the Author)

In this study, Zeng et al. used the indiscriminate dsDNA trans-nuclease activity of SuCas12a2 to selectively eliminate eukaryotic cells expressing the target RNA. Their research builds forward on earlier work (Bravo et al., Nature 2023; Dmytrenko et al., Nature 2023), where they characterized the functionality of this enzyme in prokaryotes. In the study represented by Zeng et al., the authors repurposed SuCas12a2 to target oncogenic target mRNAs for cancer therapeutics.

The authors initially demonstrated RNA and DNA trans-cleavage activity upon target RNA binding by SuCas12a2 RNPs. They then showed that chromatinized plasmid DNA can also be degraded in trans in vitro, prompting them to test whether this could be harnessed for programmed chromatin shredding. This proved to be a feasible approach for both heterologous and endogenously expressed genes, where high target abundance correlated with increased rates of cell death in several mammalian cell lines, further supported by the upregulation of multiple DNA damage markers.

To further demonstrate the therapeutic potential of SuCas12a2, the authors showed that Cas12a2-mediated transcript targeting enables selective killing of cancer cells, including those with oncogene deletions or TP53 point mutations. Using the PC9 human NSCLC cell line, they demonstrated robust in vitro killing by delivering SuCas12a2 with LNPs. Following this, in vivo experiments in mice revealed that LNP-delivered SuCas12a2 RNPs achieved modest tumor suppression and reduced metastasis.

Overall, this is a data-rich study in which the authors explore multiple contexts to demonstrate the efficacy of Cas12a2 in eliminating specific cells. The study will be of particular interest to audiences focused on cancer therapeutics and novel CRISPR-based approaches. However, in some instances (see below), the descriptions of results are not fully supported by

the data presented. Another weakness is that the applicability of the tool, as currently described, lacks sufficient foundation to be considered a reliable therapeutic strategy. For example, conclusive guidelines for selecting a gRNA and/or target transcript are largely missing and remain to be established. Additionally, it is unclear whether the differentiating power of the tool arises from an optimal PFS or is protospacer-dependent. As detailed below, incorporation of the suggested changes could make this study a good candidate for readership of Nature.

Major comments

The study could benefit from a more thorough and systematic approach towards characterizing the applicability limits of Cas12a2 before using it as a therapeutic tool. At various points in the manuscript, the gRNA designs appear to follow a hit-or-miss strategy. For example, in Figure 4, the authors make use of a single-nucleotide difference in the PFS for the Cas12a2-mediated selective elimination of cells expressing the mutant (R248Q) p53 gene. This is somewhat surprising, as the PFS was classified as rather flexible by the Dmytrenko et al. 2023 study. The authors should be more clear about whether their findings (also for other guide RNAs tested) are consistent with the proposed tolerated PFS motifs (or protospacer mismatch positions) from the Dmytrenko et al study and more importantly, how much this limits the applicability of Cas12a2 to differentiate between target RNAs with a single-nucleotide resolution.

Another example can be found in Extended Data Fig. 6e, where six different gRNAs are tested for specific targeting of a mutant p53, but the rationale for their design and evaluation of their performance are not clearly explained. The targeting efficiencies vary, and it is unclear what conclusions can be drawn from these results. For example, most guide RNA appear to be non-functional, g4 shows trans cleavage activity on both WT and mutant targets (but this is not addressed in the text), while g6 appears to perform best. Also here, it would be insightful if the authors could compare this with the Dmytrenko et al. study (e.g. were PFS distal mutations more effective?).

"Mice receiving MYC-targeting LNPs exhibited reduced tumor surface area (percentage of total liver) and decreased liver-to-body weight ratios compared to control groups (Fig. 5d; Extended Data Fig. 7b-d)". This does not seem to be supported by the data. Firstly, there are two control groups in the experiment presented in Figure 5D: PBS and a non-targeting guide RNA ("LNP NT"). Whereas the targeting guide ("LNP MYC") was significantly different from the PBS control, it does not seem to be significantly different from the "LNP NT" control, although the statistical test appears not to be performed between these two conditions. Secondly, the "liver to body weight ratios" presented in Extended Data Figure 7C appear to be very similar across the three conditions. Also here, the statistical tests are missing to substantiate these claims.

Minor comments

It is highly recommended to add a table that contains all guide RNAs (and cell lines) used in this study.

"SuCas12a2 RNPs also cleaved supercoiled plasmid DNA in trans following RNA targeting, whereas control SuCas12a2 RNPs containing non-targeting guide RNAs did not induce RNA or DNA cleavage (Fig. 1b)." The non-targeting guide RNA control, nor the RNA cleavage is present in this figure.

It would improve the readability if the authors were consistent with naming the gRNA/RNP used (currently it's a mix of "GFPg RNP", "GFP gRNA", "GFP gRNA RNP" and "GFP g"). A similar statement can be made on the inconsistent use of the Cas12 ribonucleoprotein (for example figure 1b: "Cas12a2 RNP + target RNA", figure 1c: "Cas12a2 gRNA target RNA", figure 1d: "Cas12a2-gRNA", figure 2a: "Cas12a2-gRNA RNP").

Line 84: Chromatinized plasmid appears as quite a novel term without appropriate explanations (is this a good model for chromatin) or references.

Line 121: Consider condensing the result section "Cas12a2 activity depends on Mg²⁺ concentration"

Line 149: "Transfecting gRNAs targeting ACTB (encoding β -ACTIN) or GAPDH transcripts led to cell growth arrest and expression of DNA damage markers (Extended Data Fig. 2a, b)" The authors should indicate which ACTB gRNA was used for Extended Data Fig. 2b. Did the authors see a similar trend in the upregulation of these DNA damage markers, upon using different 'strengths' of ACTB gRNAs? Why did targeting the transcript of the house-keeping gene GAPDH result in almost full confluence similar to the non-target control?

Line 153: "GFP expression increased within 4 hours after transfecting ACTB gRNAs, peaking at around 24-48 h (Fig. 2f)". The plateau is reached around 24h; it is unclear why the authors are mentioning such a wide range, as the x-axis ends at 36h. Was the p21-GFP signal declining after 48h?

Line 159: "For example, CCNE1 and MYC are frequently amplified in various cancers." The provided reference does not mention MYC. Please provide an appropriate reference.

Line 168: "Whereas some gRNAs (gRNA 3, 4 and 5) induced cell growth defects in both untreated cells and Dox-treated cells, gRNA 1 and 2 induced growth defects selectively in Dox-treated cells (Extended Data Fig. 3e). These observations suggested that a targeting window exists to distinguish between high and low levels of the same transcript". The different lines in these graphs are very hard to distinguish/compare. It is unclear why gRNA that provoke cell death in the -Dox

condition do not perform better (and sometimes appear to perform worse) in the +Dox condition.

For the results presented in Figure 4C, the authors do not discuss the "GFP g" results. Was this used as a positive control and are both cell lines making use of a GFP that was knocked in-frame of the (p21-encoding) CDKN1A gene, as described on page 6? It is a bit confusing, as the next section mentions that the RPE1 p53 R248Q cell line expresses mCherry rather than GFP (as the authors use it to differentiate the two cell lines in Figure 4D) and the methods do not provide any details either.

The results presented in Extended Data Figure 4D show that the competition experiment included a positive control (i.e. a guide anticipated to kill both cell lines by targeting ACTB), but was not described in the corresponding results section, nor quantified in Figure 4E.

Please double-check the formatting of the error bars and their description in the legend; they are sometimes absent.

The authors might want to acknowledge and/or discuss the recent pre-print published by Jorik et al. on targeted LbuCas13a-mediated cell elimination?

Referee #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #5

(Remarks to the Author)

This was a very easy paper to review, and I want to briefly explain why.

First, cancer-selective therapies have been and will always be *the* holy grail to achieve precise and durable treatment with minimal side effects. To this day, this has not been realized.

Second, mutant p53-selective therapies have also been and will continue to be *the* biggest holy grail because >50% of *all* human cancers harbor TP53 mutations, and they are almost *the* pathognomonic genetic lesion in often recalcitrant tumor types (e.g. ovarian cancers). To this day, this has not been realized either, but *many*, *many* people have tried.

Third, most *direct* "mutant p53-selective" therapeutic attempts have only focused on 1-2 "hotspot" p53 mutations, thereby ignoring hundreds of p53 mutations (most of which remain poorly understood and understudied). Most of these have failed for many reasons, including "off-target" effects whereby e.g. small molecules have later been found to target other proteins instead of mutant p53 proteins themselves. While some exciting developments in this space are seeing some success (e.g. TP53-Y220C-targeting small molecules), I would strongly argue that, to this day, this particular holy grail has not been realized either.

Fourth, most of the *indirect* "mutant p53-selective" therapeutic attempts, whereby other factors that support the malignant properties of mutant p53 proteins or the fitness of mutant p53-expressing cancer cells in still mechanistically obscure ways, are just too toxic to implement in the clinic (e.g. HSP90 inhibitors). Thus, I would also strongly argue that, to this day, this particular holy grail has not been realized either.

Fifth, even if all of the above advancements were successful and went into the clinic, the reality is that tumors will acquire treatment resistance. For targeted therapies that e.g. inhibit or target specific mutant oncogenes (or, in this case, mutant tumor suppressor proteins like hotspot mutant p53 proteins), this often happens by direct mutation of the target itself, which e.g. ablates drug binding and selectivity. Historically, this has required follow up drug development campaigns to find molecules capable of targeting the now-mutant oncogenic driver (e.g. EGFR, EML4-ALK, BCR-ABL, and the list goes on), which takes years and millions of dollars, and always fail to reach the patients that needed it right at the moment that their tumors became resistant. And for the ones that now benefit from 2nd, 3rd, 4th, or nth generation molecules, the clock is already ticking, yet there hasn't been a way to proactively and prospectively target the resistant disease that will inevitably arise later. Thus, I would also strongly argue that, to this day, this particular holy grail has not been realized either. But it clearly needs an approach that is amenable to prospective design and testing, rational (re)programming and customization, and rapid manufacturing and therapeutic deployment.

This study effectively *shreds* each of the above problems in a way that tremendously excites this Reviewer who has been working on (mutant) p53, including therapeutic restoration, since graduate school. It presents the most compelling strategy I have seen thus far, aside from another yet-unsolved holy grail: the now-possible therapeutic correction of genetic mutations, with mutant p53 alleles being high up in that list for this Reviewer. Most importantly, their strategy has virtually unlimited (re)programmable potential and multiplexability, even longitudinally speaking, as it is a *hit-and-run* strategy that exploits programmable RNA and Cas proteins in RNPs.

As such, I firmly believe that this paper should be immediately accepted, at least in principle, after the authors incorporate more discussion (including some limitations) and, most importantly, key references that are missing, particularly those related to landmark in vivo studies (reviews should not suffice in this case for a number of key studies).

Below are some general comments in this direction:

1. What I consider to be *the* key references in the field of therapeutic p53 restoration are completely missing:

Martins, C. P., Brown-Swigart, L. & Evan, G. I. Modeling the therapeutic efficacy of p53 restoration in tumors. *Cell* 127, 1323–1334 (2006)

Ventura, A. et al. Restoration of p53 function leads to tumour regression in vivo. *Nature* 445, 661–665 (2007)

Xue, W. et al. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* 445, 656–660 (2007)

Feldser, D. M. et al. Stage-specific sensitivity to p53 restoration during lung cancer progression. *Nature* 468, 572–575 (2010)

Junttila, M. R. et al. Selective activation of p53-mediated tumour suppression in high-grade tumours. *Nature* 468, 567–571 (2010)

Christophorou, M. A. et al. Temporal dissection of p53 function in vitro and in vivo. *Nature Genet.* 37, 718–726 (2005)

2. There is no discussion about the fact that the most common configuration of mutation of TP53 in human cancers is a point mutation (e.g. a hotspot mutant) over a segmental deletion at 17p that includes TP53 and several other genes. This means that cancer cells express only one type of mutant p53 mRNA molecule from the single TP53 mutant allele present; as such, they are one deletion away from becoming resistant to their therapeutic approach, which is worth discussing.

3. Related to the above, some human tumors have two different (missense) mutant TP53 alleles, so targeting these would require multiplexing to hit each of the two mutant mRNAs. Otherwise, I would predict that they wouldn't be sensitive. This is not an experiment that is required, as I think their R248Q and R175H targeting specificity experiment is quite convincing. But this is worth discussing.

4. Their statement "unintended p53 activation... duplication (ref. 14-16)". This statement is incomplete. There is a substantial body of work related to how unintended p53 activation in cells of and within the GI tract that can lead to radiation-induced gastrointestinal syndrome due to p53-regulated cell death (not apoptosis) (e.g. . It is also reasonably well-established that certain cells, particularly of the hematopoietic system, can be quite sensitive to p53 over-activation, so this is worth discussing, too (PMID: 20019247), in the context of those introductory sentences.

5. It is also worth discussing that their liver tumor modeling experiment employed overexpression of human wild type MYC (correct?). In this regard, I thought it would be interesting and important to discuss the fact that there are missense point mutations in MYC that evade p53-dependent apoptosis, which would benefit their discussion in at least two ways. First, the authors could discuss that their approach could also be used to target mutant MYC alleles that are also tumor-specific and over-expressed, bypassing any issues with potential "on-target off-targeting" activity in normal cells that may express higher-than-average levels of MYC, and second, this would be the perfect scenario where they could connect their two main proof-of-concept studies.

6. Unless I missed it, I thought it was a bit of a missed opportunity to not perform molecular analysis on their various mutant-specific targeting experiments. For instance, a set of simple RNA-seq experiments would've been interesting to see if p53-related pathways are now being reinstated upon mutant p53 transcript targeting (e.g. pathways that the wild type protein typically would suppress, which are now active in the presence of the mutant, but then no longer active --> is that enough to reinstate parts of the tumor suppressive pathway that are inactive in the mutant state, but are now back to baseline or reinstated)? But, honestly, I see no immediate or urgent need for this type of experiment because the responses are impressive and the specific mechanism beyond the chromatin shredding one is not critical to make the point here.

Overall, this manuscript is very innovative, timely, exciting, and important, and it is definitely a "Nature paper". It deserves to be published at the highest level, and soon.

Points 1-5 above are the most important ones for me.

P.S. — one other interesting application would be to selectively eliminate human (or mouse) embryonic stem cells from a therapeutic culture that are known to acquire TP53 mutations over time (e.g. see <https://www.nature.com/articles/nature22312>).

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have done a commendable job of addressing the concerns raised in the first round of review. In particular, I

appreciate the addition of the matching control for the EGFR-targeting cell-killing assay, which strengthens Figure 3, as well as the data demonstrating protospacer-targeted recognition of a single point mutation.

Two remaining concerns must be addressed:

1. The p53 cell-killing experiments still lack expression-level controls for two of the three targeted mutants. Because cell killing is dependent on expression levels, the authors must demonstrate that the expression levels of R280K and E285K are equivalent to the R175H control. Furthermore, they need to explicitly state what the wild-type control is. If wild-type p53 is being used as a control, its baseline expression is substantially lower than that of the targeted mutants, making it inappropriate for this comparison; in that case, I suggest removing these data. In lines 232–235, the text implies that the wild-type, R248Q, R280K, and R175H variants have comparable expression levels. However, no data are shown for R280K, and Extended Data Figure 4f indicates that wild-type expression is reduced by at least 20-fold. An expression-level control for E285K is also missing.
2. I remain concerned about the lack of appropriate controls for the in vivo tumor-killing experiments. The authors argue that demonstrating specificity is unnecessary because the focus is on therapeutic potential; however, they must still definitively show that the observed activity is driven by the proposed mechanism of action. Given that p53 is central to this study, it seems inappropriate to conclude the experiments when comparing the treatment to the non-target control yields a p-value of 0.10 for p53.

Referee #2

(Remarks to the Author)

Overall, this reviewer is satisfied with this revision and finds the proposed cancer-specific, reprogrammable targeting strategy both innovative and promising for addressing long-standing challenges associated with p53- and drug-refractory cancers.

In the revised manuscript, the authors have included new experimental data and text revisions to address my main concern regarding targeting selectivity. While an ideal assessment of mismatch tolerance would involve systematic single-nucleotide perturbations of a defined active guide RNA for Cas12a2, the new guide-tiling experiments across two target sites (28 guides total) in Fig. S4 provide a reasonable and informative alternative. The observation that only a small subset of guides (1/14 and 6/14) achieved robust targeting underscores the complexity of guide design and the need for empirical optimization. Importantly, the two strongest guides showed clear selectivity between cancer and wild-type target transcripts, indicating that placing the mismatch at the PFS may be a favorable design strategy. The author also acknowledged in the main text that design rules for effective Cas12a2 guides remain incompletely defined, and that PFS requirements and mismatch discrimination in mammalian cells may differ from those in bacteria and be influenced by multiple factors.

Additional new experiments include the use of isogenic cell lines expressing mutant and wt P53 at comparable levels, effectively ruling out the caveat that differential activation arises from difference in target transcript abundance rather than the 1nt mutation in cancer mRNA. The authors also note in the main text that escape of Cas12a2 killing could in theory occur through downregulation of target mRNA expression. Together, the revisions have adequately addressed my concerns.

Referee #3

(Remarks to the Author)

The authors have thoroughly addressed my and the other reviewers' comments. I have no further recommendations for improvement.

Referee #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #5

(Remarks to the Author)

The authors have addressed all the points I brought up in my peer review and, in my opinion, convincingly addressed all other critical points brought up by the other reviewers. I believe this study is quite innovative, thorough, important, and fully deserving candidate for publication in Nature. It will start a new wave of efforts in the field, including (but not limited to) cancer-focused therapeutic studies. It will push MAJOR ideas and efforts in the field of p53, which remains prime and urgent from a therapeutic point of view (e.g., <https://www.nejm.org/doi/full/10.1056/NEJMoa2508820>). It is also fundamentally and mechanistically interesting and thorough, particularly with their new data on the impact of PFS and their detailed tiling studies. Congratulations to the authors for an excellent study, and I look forward to seeing the final product.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)

I am satisfied with the revision and recommend publication.

Referee #3

(Remarks to the Author)

I'm pleased to see that the authors have restored the data and revised the text accordingly, after it had been removed from the previous (revised) version of the manuscript, more specifically:

- The non-targeting control data and targeting data (Fig. 1b)
- The (minor) effect of the GAPDH sgRNA on cell confluence (Extended Data Fig. 2b)

One last remaining issue is that in the 'Original gels' file that was part of the submission, panel 1c and panel 1d are mislabeled.

Referee #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

Response to referees' comments

Referee #1 (Remarks to the Author):

In this manuscript titled "Selective Elimination of TP53 Mutant Cells by Transcript-Activated Chromatin Shredding", the authors present in vitro and in vivo data on the selective elimination of cells containing transcripts that match the guide RNA in a recently described CRISPR system, Cas12a2. Cas12a2 detects target RNA and degrades both the target RNA and trans double-stranded DNA without sequence preference. The data presented are consistent (though not fully supportive) with a model in which Cas12a2 can be used to deplete cells harboring mutant genes. Although the authors provide several examples, including GFP targets and various oncogenes, the primary focus is on TP53, where mutations may induce tumor formation but the mutant protein itself cannot be directly targeted with small-molecule drugs. Instead, the proposed strategy is to target the transcript produced from the mutant gene. The most intriguing candidates are single-point mutations that presumably maintain normal p53 protein levels but nevertheless promote tumor initiation and growth.

The in vivo examples are potentially exciting, particularly if they can indeed target lung tumors using a recently developed LNP formulation. However, I have major technical concerns and cannot recommend publication until these issues are addressed with additional experiments. My concerns fall into two major categories:

- (1) Lack of adequate controls to support the claim that cell elimination is based on transcript sequence rather than transcript abundance in most experiments.*
- (2) Low statistical power of the in vivo data.*

We thank this reviewer for their thorough evaluation and constructive criticism. We are pleased that the reviewer finds our manuscript to be "potentially important" and "of broad interest." We have carefully addressed each concern with new experiments and substantial text revisions as detailed below. First, we have carefully controlled expression levels in our experiments by either creating isogenic cell lines with comparable levels of wild-type and mutant transcripts, or by comparing expression levels with qPCR. Second, we have thoroughly tested single-nucleotide discrimination by adding a systematic mismatch dataset in human cells (see our responses to Referee #2 and #3 as well), including guide RNA tiling, dose-response and GR50 quantification at two TP53 loci (R248Q and R280K). Third, for the *in vivo* studies, we now report full statistical details and have also added discussion of strategies that may further improve therapeutic efficacy.

Specific Comments

- 1. While I appreciate the authors' transparency regarding the need to treat mice six times with LNP deliveries over several days due to low targeting efficiency (only ~10% of tumor cells receive them), they should provide data confirming that the mouse lung and other physiological parameters remain normal after repeated treatments, as LNPs may accumulate elsewhere.*

We agree that this is an important point. In the revised manuscript, we have included representative histopathological images of lung and additional organ tissues from treated animals (Extended Data Fig. 9c), evaluated by board-certified histopathologists. These images show no obvious pathological changes. We have also cited prior studies using the LNP formulation that include additional toxicology analyses.

2. Figure 5d (in vivo data): More data points are needed to support any strong claim of selective tumor cell elimination. The difference between PBS and LNP-MYC appears marginal, with only a single significance marker (unclear which statistical test was used and how many animals were included). This is not convincing. Even more striking is that the difference between LNP-NT and LNP-MYC is even smaller, and no statistical measure is provided. These groups must differ for the authors to claim selectivity. Additional animals are needed before making such claims.

We thank the reviewer for these important comments and agree that the *in vivo* data should be interpreted carefully. In the revised manuscript, we now include statistical comparisons between all treatment groups in the liver and lung tumor experiments and have clearly indicated the number of animals and statistical tests used in the figure legend. Specifically for the liver tumor treatment experiment, tumor burden differs significantly between PBS and LNP-MYC ($p = 0.014$) and between LNP-NT and LNP-MYC ($p = 0.031$), whereas PBS and LNP-NT are not significantly different ($p = 0.340$). These additions clarify the statistical analysis and support the reported differences between treatment groups.

5. Figure 5f: The only time point showing a significant difference between PBS and LNP-p53 R248Q is at 15 days. Other time points (11 and 22 days) show no difference. More animals are needed before making definitive claims about selectivity.

We thank the reviewer for raising this important point and agree that the statistical analysis should be clearly presented. We note that this comment appears to relate more to therapeutic efficacy (the extent to which the treatment reduces tumor burden) than selectivity (the ability of Cas12a2 to distinguish mutant from wild-type transcripts). For additional data addressing selectivity, please see our responses to Comments 3 and 6, as well as our responses to Reviewers #2 and #3.

In the revised manuscript, we now include statistical comparisons between all treatment groups and report the corresponding p values in the updated figure 5. At day 15 for the early-stage tumor treatment test, tumor burden differs significantly between the PBS and LNP-p53 R248Q treatment groups ($p = 0.021$). The comparison between LNP-NT and LNP-p53 R248Q shows a trend toward reduced tumor burden but does not reach statistical significance ($p = 0.105$), whereas PBS and LNP-NT groups are not significantly different ($p = 0.721$). These results are consistent with a treatment-dependent effect, although the overall magnitude of tumor suppression *in vivo* was modest.

Importantly, additional analyses support the proposed mechanism of action. Residual tumors collected at the experimental endpoint after LNP-p53 treatment showed reduced levels of the targeted TP53 mutant transcript (by qRT-PCR; Extended Data Fig. 9g), consistent with preferential elimination of cells expressing the target transcript. This also suggests that downregulation of the target transcript may represent a potential resistance mechanism. We have added this interpretation to the Discussion and now note that further improvements in therapeutic efficacy will likely require optimization of delivery systems, multiplexing and engineering of Cas12a2 variants with enhanced activity.

3. Continuing with Figure 5d: The authors should perform experiments targeting tumors that do not carry the targeted mutation to demonstrate selective elimination of TP53 mutant cells. While they show some cultured cell data in Figure 4, the absence of such *in vivo* controls makes it difficult to accept their claim of selectivity.
6. ...Additionally, the authors should include data from tumors lacking the targeted p53 mutation before claiming selective *in vivo* tumor reduction via the proposed mechanism.
7. Overall, the *in vivo* data require additional evidence to support the proposed mode of action.

We appreciate the reviewer's concern regarding selectivity and agree that this is a critical point for evaluating the proposed mechanism. We believe that the key question of mutation selectivity is most directly addressed in controlled cellular systems where transcript levels and experimental conditions can be precisely matched. Accordingly, we performed comprehensive experiments under conditions where mutant and control transcripts were expressed at comparable levels in isogenic cell lines.

Specifically, EGFRdel-, p53 R248Q- and p53 R280K-targeting guide RNAs were evaluated using dose-response experiments designed to quantitatively assess selectivity (Fig. 3 and 4; Extended Data Fig. 4) (see also responses to Comments 10 and 11). Under these matched conditions, the R248Q-targeting guide exhibited approximately 100-fold greater potency against p53 R248Q transcripts compared to p53 R175H control transcripts expressed at similar levels. For guides targeting EGFRdel and p53 R280K, we observed even stronger discrimination, with no detectable DNA damage induction in control cells even at high guide RNA concentrations.

Together, these results demonstrate robust mutation selectivity of Cas12a2 when transcript abundance is controlled. These experiments provide clear mechanistic evidence for selective targeting of mutant transcripts. The *in vivo* experiments in Figure 5 were intended to evaluate therapeutic feasibility, while mutation selectivity is more directly addressed by the controlled cellular experiments described above.

4. Figure 5d: The manuscript does not describe what the NT gRNA represents.
18. Non-target guides: I could not find information about what was used as non-target guides. There are many experiments with different targets in this manuscript. Did the authors use the same non-target guide for all experiments, and if so, why was it chosen?

We thank the reviewer for pointing out this omission. We have now added a comprehensive table listing all guide RNA sequences used in this study (Supplementary Table 1), including the NT gRNAs. For cell-based experiments, we used a gRNA complementary to zsGreen mRNA as the NT control, whereas for *in vivo* experiments we used a gRNA complementary to GFP mRNA. Both guides target exogenous sequences that are absent from the cells and tissues analyzed in these experiments. Importantly, we also verified that these NT guides do not induce detectable DNA damage responses or growth inhibition in cells lacking the corresponding exogenous targets (Fig. 2d; Extended Data Figs. 1a and 2d). These results support their use as appropriate non-targeting controls in the experiments described in this study.

8. Extended Data Figure 1: 550 transcripts per cell is a relatively high transcription level, yet the authors observed only modest growth defects. This suggests the approach may require unusually high RNA expression. If this is a function of RNP concentration, the authors should characterize the transcript copy number required for cell killing at higher RNP concentrations. The statement “Control cells with no GFP expression were unaffected” may hold at low RNP concentrations, but what about higher concentrations where fewer target transcripts might suffice?

We thank the reviewer for raising this important point. To evaluate the GFP expression levels in cells used in this study, we performed RNA-seq analysis of parental HEK293 cells and HEKGFP Mid cells (used in Fig. 2a–d and Extended Data Figs. 1a–e). We ranked transcript abundance across the protein-coding transcriptome (RNA-seq data provided in Supplementary Table 5). The GFP transcript in HEKGFP Mid shows a CPM (counts per million) of 678. Although this cell line contains approximately ~1,100 GFP transcripts per cell, its GFP transcript's CPM ranks 140th among all protein-coding transcripts in this cell line, indicating that the reporter represents a highly expressed but physiologically relevant target rather than an extreme outlier.

Importantly, we performed additional dose-response experiments in control cells lacking GFP expression (data added in Extended Data Fig. 1b). Across the tested RNP concentration range, we observed no detectable growth inhibition, indicating that cells lacking the target transcript remain unaffected even at higher RNP doses.

We agree that Cas12a2-mediated killing is likely influenced by multiple factors, including gRNA activity, target transcript abundance, and RNP concentration. For example, ACTB and TP53 R280K guides targeting different positions within the same transcript induce varying levels of DNA damage responses (Extended Data Fig. 2d; 4c), illustrating the strong influence of guide-dependent activity. Establishing quantitative design rules that integrate these parameters will be an important direction for future work.

9. Extended Data Figure 1f: The authors state that DNA cleavage in trans occurred at 0.1 mM Mg^{2+} , but the gel shows that most trans DNA remains uncleaved even after 1 hour. The data do not align with the claim. Quantification is needed (e.g., “2% of DNA was cleaved”), and replicates should be included to confirm reproducibility.

We have now added quantification of the gels and calculated cleavage rates. Gel quantification shows that ~30% of *trans* DNA was cleaved at 0.1 mM Mg^{2+} after 1 hour.

10. Figures 3b–e: The comparison between mutant and wild-type transcripts was made using two different cell types (RPE1 vs. PC9), which is not convincing. The authors did not compare transcript levels between these cell types. Given that GFP targeting shows dependence on transcription levels, they must demonstrate that transcript levels are comparable here.

19. Figure 3: This figure as a whole is problematic because the authors did not control for RNA levels. Unless they can demonstrate that transcript levels are similar between PC9 mutant and RPE1 wild type, I suggest removing this figure. Without such evidence, they cannot claim that cell elimination is selective for the targeted mutation.

We agree with the reviewer that it is important to control for differences in cellular context or transcript abundance. To address this directly, we established RPE1 cells expressing EGFR WT and EGFR E745_A750del by lentiviral transduction of the corresponding EGFR WT and mutant transgenes using the same MOI and transduction conditions. We then confirmed that EGFR transcript levels were comparable between the two lines by RT-qPCR (Extended Data Fig. 3g). Using these expression-matched RPE1 models, we tested the EGFRdel-targeting gRNA and observed significant growth inhibition (GR50 = 0.055 nM) and robust DNA damage signaling in RPE1 EGFR E745_A750del cells, whereas RPE1 EGFR WT cells showed no detectable growth inhibition or DNA damage (data in revised Fig. 3). Importantly, this selectivity was maintained across a dose-response series, and no growth inhibition or DNA damage was observed in the EGFR WT line even at the highest gRNA concentration tested. These new data support the conclusion that sequence-specific recognition of the mutant EGFR transcript, rather than differences in cell type or expression level, drives the observed phenotype.

11. Figure 4e: The competitive growth comparison between mutant and wild type does not account for transcript abundance differences. Extended Data Figure 4f suggests wild-type cells have 20-fold fewer transcripts. If true, the enrichment of wild type in competitive growth assays cannot be attributed to mutant-selective cell elimination.

12. When comparing R175H as a control, the authors observe similar transcript counts as R248Q yet see a growth phenotype only for the targeted mutation. This appears to be the only experiment where mutant and wild type are compared side by side. Why did they not perform a competition assay, which could be easily done via amplicon sequencing?

We thank the reviewer for raising this important point regarding transcript abundance. To more directly address this issue, we performed side-by-side dose-response experiments comparing p53 R248Q and control R175H cells with similar TP53 expression levels. As shown in Fig. 4e, the R248Q-targeting guide exhibits approximately ~100-fold greater cell growth inhibition potency against p53 R248Q cells compared to p53 R175H cells. Consistent with this result, the guide also induces substantially higher levels of DNA damage in p53 R248Q cells, while causing no or minimal DNA damage in WT or p53 R175H cells (Extended Data Fig 4g). These findings support mutation-dependent selective targeting rather than differences in transcript abundance. In addition, we have moved the competition experiment involving wild-type cells to the supplementary materials, and revised the interpretation of the competition result in the main text as showing that Cas12a2-damaged cells can be depleted from a heterogeneous population.

13. Extended Data Figure 5a: The authors claim that “trans DNA cleavage by Cas12a2 RNPs containing R248Q gRNA was specific to the R248Q mutant transcript,” but the figure shows that most trans DNA remain uncleaved, indicating low Cas12a2 activity. Replicates and quantification are needed.

We thank the reviewer for this comment. We have now added quantification and calculated cleavage rates, showing that in the presence of the mutant R248Q target RNA, *trans* DNA cleavage occurs at a rate ~28-fold higher than with the WT control. After 1 hour ~40% *trans* DNA was cleaved in the presence of the mutant target.

14. The statement “These results indicate that Cas12a2 RNPs can precisely target TP53 mutant cells with high selectivity and minimal side effects in healthy cells” is problematic. Mutant cells appear healthy when treated, so calling controls “healthy” is misleading. More importantly, the authors must show that wild-type cells with equivalent transcript abundance are not eliminated. This is critical because they claim, “Taken together, these results demonstrate that SNVs spanning TP53 mutant transcripts can be exploited for Cas12a2-mediated selective cell killing.” Selectivity must be demonstrated convincingly.

We thank the reviewer for this thoughtful comment. We have strengthened the experimental support for selective targeting by performing additional dose-response experiments at the p53 R280K and R248Q loci (Fig. 4 and Extended Data Fig. 4). In these experiments, mutant p53-expressing cells were compared with control cells expressing wild-type or control p53 R175H transcripts at matched expression levels, allowing direct assessment of sequence-dependent selectivity. These analyses demonstrate substantial differences in potency between target and control cells, supporting selective Cas12a2 activity based on transcript sequence rather than transcript abundance is controlled.

In addition, we have removed the term “healthy cells” throughout the manuscript and now refer instead to “cells expressing wild-type/control transcripts.”

We have also moderated the broader claim regarding selectivity. The revised text now reads: *“Together, these results demonstrate that in mammalian cells, Cas12a2 can discriminate point mutations located either within the PFS or within the protospacer region of the target transcript.”*

15. The sentence “Cas12a2 showed trans-cleavage selectivity in vitro with two of the gRNAs (gRNAs 4 and 6) targeting the M246I mutant transcript, suggesting a single mismatch between the gRNA-target duplex can decrease Cas12a2 activity” is unclear. Why does this observation suggest that a single mismatch reduces activity?

gRNAs 4 and 6 show strong activity on the M246I mutant target but reduced or absent activity on the WT target (which contains a 1-nt mismatch at the mutation site). This differential activity pattern suggests that single mismatches in the spacer reduced Cas12a2 activation. We acknowledge that gRNA 4 shows limited selectivity as it also led to *trans*-cleavage in the presence of the WT transcript, hence we removed gRNA 4 from the sentence. In addition, our new data on R280K gRNAs also show that 1-nt mismatches at different spacer locations reduce Cas12a2 activity (Extended Data Fig 4). Together, these results indicate that Cas12a2 activation is sensitive to single-nucleotide mismatches in mammalian cells.

16. “Cas12a2 targeting with one of the selective gRNAs, M246I gRNA 6, induced growth defects in NCI-H23 cells (p53 M246I) but not in U2OS cells (p53 WT).” I have similar concerns here. The authors are comparing different cell types, which does not rule out the possibility that the observed difference arises from cell-type-specific factors or differences in transcript abundance.

We agree with the reviewer that expression levels should be adequately controlled. To address this, we measured TP53 expression levels in NCI-H23 and U2OS cells. Extended Data Fig 6f shows that these two cells have similar TP53 expression levels as detected by RT-qPCR.

17. It would be helpful for the authors to explain why they target the mutation through the PFS rather than through the protospacer.

We thank the reviewer for this helpful suggestion. In the revised manuscript, we now include screening data in which mutation sites at p53 R248Q and R280K were tiled with 28 gRNAs per site, including gRNAs targeting the mutation through the PFS and those targeting the mutation within the protospacer. From the screen, we identified gRNAs capable of inducing DNA damage in target cells through both targeting modes, but the strongest gRNAs at both loci happen to target through the PFS. We also note in the revised text that mutation-dependent targeting through short recognition motifs has precedent in CRISPR systems. In particular, allele-specific targeting through PAM-disrupting mutations has been used for Cas9-based approaches, and we now cite representative examples.

Summary:

This is a potentially important manuscript of broad interest; however, there are numerous major concerns that must be addressed before publication.

Referee #2 (Remarks to the Author):

Zeng et al. report an innovative strategy for selective cancer cell killing by harnessing Cas12a2, an RNA-activatable nuclease that cleave trans-DNA non-specifically upon sensing cognate RNA transcripts. Building on two earlier studies by others that characterized Cas12a2's RNA-activated, trans-nucleolytic biochemical activities, this work provides a logical and timely extension of Cas12a2's usage into mammalian cells. The authors first show that once activated by a target reporter and endogenous transcript, SuCas12a2 triggers acute DNA damage and cell-cycle arrest in cultured human cells. By exploiting signature mutations in cancer-associated transcripts, including an EGFR in-frame deletion and the most common p53 SNVs, they further demonstrate that Cas12a2 can discriminate mutant from WT transcripts with selectivity, leading to potent killing or proliferation arrest across multiple cancer cell lines. Finally, and impressively, LNP-mediated delivery of Cas12a2 targeting the p53 R248Q mutation enables in vivo tumor burden reduction in MYC-driven liver cancer and lung cancer xenograft models.

Overall, this study is conceptually bold, novel, technically sound, and presented with clarity. The central intellectual advance, selective cancer cell killing, is arguably the most appropriate and compelling application of Cas12a2 in eukaryotic settings. Importantly, the work helps address a major unmet medical need in oncology, namely the long-standing difficulty of targeting undruggable oncogenic signatures such as TP53 mutations. Given the strength of the findings, their clinical relevance, and the potential — with continued development — to establish a new paradigm in programmable and precision cancer therapeutics, this study will be of broad interest to the research community.

Below I outline several substantive concerns and recommendations, including one major issue (the lack of a systematic single-nucleotide mismatch discrimination analysis, see below for details) that must be addressed to ensure the platform's rigor and generalizability.

We thank this reviewer for their thoughtful and encouraging evaluation of our work, and recognition that our work has potential to "establish a new paradigm in programmable and precision cancer therapeutics." We appreciate the reviewer's constructive suggestions aimed at strengthening the rigor and generalizability of the study. In particular, we have carefully addressed the major concern regarding systematic characterization of single-nucleotide mismatch discrimination in human cells by performing mismatch-tiling analyses at two independent TP53 loci, enabling evaluation of both PFS-based targeting and protospacer mismatch discrimination in mammalian cells. We believe these additions and revisions substantially strengthen the manuscript and address the reviewer's concerns.

1. To establish Cas12a2 as a generalizable cancer killing platform, the manuscript would benefit from a more systematic investigation of 1-nt mismatch tolerance in human cells. While the p53 R248Q targeting data in Fig. 4, 5, and S6a are compelling —demonstrating that placing the oncogenic mutation within the PFS region confer strong selectivity—it is unclear what fraction of oncogenic mutations can realistically benefit from this A-rich PFS discrimination strategy. It is likely only a modest subset. As shown in Fig. S6i, 73% of TP53 mutations have nearby adenines that could serve as PFS motifs, meaning that in most cases the pathogenic nucleotide would reside within the guide region rather than within the PFS itself. For these more common scenarios, defining Cas12a2's single-nucleotide mismatch discrimination rules within the guide region is essential, particularly because the original 2023 bacterial study reported promiscuous mismatch tolerance. This underscores the need to rigorously characterize mismatch discrimination in human cells, where therapeutic applications demand much higher precision. Such insights would also inform users in designing effective gRNAs. I suggest that the authors conduct a more systematic analysis in which one or more TP53 loci are tested with 1-nt mismatches introduced at multiple positions across the protospacer. Such data would provide crucial insights into targeting specificity and significantly strengthen the platform's generalizability.

In the revised manuscript, we performed a systematic mismatch-tiling analysis at two independent TP53 loci (R248Q and R280K) in human cells (Extended Data Fig. 4). For each locus, we generated 28 guides tiled across the mutations and measured DNA damage induced by these guides in target mutant cells. We then determined dose-response relationships for two best hits, R248Q gRNA3 and R280K gRNA1, both targeting the mutation through PFS, in target mutant cells and control cells. Both gRNAs induced strong growth inhibition (nanomolar-range GR50) and DNA damage in target cells but no or minimal effects in control cells, consistent with strong selectivity (Fig 4). This suggests the PFS-targeting strategy can be highly selective. In addition, we characterized mismatch sensitivity within the spacer. Results at the R280K locus show that single-nucleotide mismatches within the spacer can also reduce Cas12a2 activity (Extended Data Fig 6). In the revised manuscript, we have reorganized the TP53 Results section into two parts: one describing the PFS-based targeting strategy and a second describing gRNAs with mismatches within the protospacer. We believe this expanded dataset provides clear characterization for mismatch sensitivity in human cells. At the same time, we acknowledge that mismatch discrimination in mammalian cells may differ from those characterized in bacterial systems, and design rules for effective Cas12a2 guides remain incompletely defined. We have therefore expanded the Discussion to note these points and

emphasize that guide RNA screens and systematic analyses will need to be performed in human cells to establish generalizable Cas12a2 guide design principles.

2. Along the same line, the description of the PFS requirement for SuCas12a2 could be clarified to better orient the readership. In the current manuscript, it is only briefly mentioned on p.9 in reference to Fig. 4, “SuCas12a2 recognizes an adenine-rich PFS site adjacent to a targeted RNA sequence”. However, the original study that defined the PFS (PMID: 36599979; 5'-GAAAG 3' PFS) provides a more precise specification of its length and nucleotide identities. Introducing this PFS information more explicitly and much earlier in the manuscript (i.e., before any RNA-targeting experimental results are described) would help readers better understand the basis for the RNA-targeting designs that follow.

We thank the reviewer for this helpful suggestion and agree that the PFS requirement should be introduced in more detail. In the revised manuscript, we now introduce the SuCas12a2 PFS requirement in the first paragraph of the Results section to clarify the basis for our gRNA designs. We have also added a schematic illustrating the PFS in Fig. 4 to help readers better understand how the p53 mutant-targeting gRNAs relate to PFS recognition.

3. Also related to targeting selectivity, the following data interpretation for Fig. S6f (p.11) is a bit misleading: “Cas12a2 showed trans-cleavage selectivity in vitro with two of the gRNAs (gRNAs 4 and 6) targeting the M246I mutant transcript, suggesting a single mismatch between the gRNA-target duplex can decrease Cas12a2 activity (Extended Data Fig. 6f)”. However, this conclusion appears inconsistent with data shown in Fig. 6f, where genomic DNA shredding by Cas12a2 is measured in the presence of WT versus mutant p53 target transcripts. g2, g3, and g5 induced similarly minimal or no degradation for both targets. g1 shows modest degradation for the mutant but not the WT transcript. Most importantly, g4 triggered strong degradation for both targets—although cleavage was somewhat higher for the mutant, the WT transcript still induced substantial degradation. Thus, describing g4 as “selective” based on the slightly decreased activity is technically not wrong, but quite misleading. Overall, g6 is the only guide that demonstrates clear and comfortable selectivity. The interpretation of Fig. S6f should be revised accordingly to more accurately reflect the data.

Again, only one out of six guides demonstrated meaningful selectivity, underscoring the critical need for a comprehensive characterization of Cas12a2's 1-nt mismatches discrimination across multiple targets using in human cells a full panel of mismatched guides. This is a major concern that shall be dealt with prior to publication.

We thank the reviewer for this careful assessment. We have revised the text to mention only M246I gRNA6 as the guide in this set that shows clear selectivity. We agree that a more systematic characterization of mismatch discrimination would be useful. To address this, we performed mismatch-tiling analyses at two independent TP53 loci (R248Q and R280K) (see response to Comment 1).

4. For clarity, it would be helpful if the figure legends specified whether the error bars represent SD or SEM. Please add this information consistently across all figures to improve readability.

We have clarified error bars throughout the manuscript for consistency.

5. The statistical analysis for the LNP-MYC sample in Fig. 5d should be performed relative to the NT control, as is done consistently across all other panels, rather than relative to PBS. Using PBS here may confound interpretation, as NT is the proper statistical inference.

We thank the reviewer for this suggestion and agree that comparison to the LNP-NT group provides the most appropriate statistical control. In the revised manuscript, we have included statistical comparisons between the LNP-MYC and LNP-NT groups (Fig. 5d), in addition to the comparison with PBS controls.

Referee #3 (Remarks to the Author):

In this study, Zeng et al. used the indiscriminate dsDNA trans-nuclease activity of SuCas12a2 to selectively eliminate eukaryotic cells expressing the target RNA. Their research builds forward on earlier work (Bravo et al., Nature 2023; Dmytrenko et al., Nature 2023), where they characterized the functionality of this enzyme in prokaryotes. In the study represented by Zeng et al., the authors repurposed SuCas12a2 to target oncogenic target mRNAs for cancer therapeutics.

The authors initially demonstrated RNA and DNA trans-cleavage activity upon target RNA binding by SuCas12a2 RNPs. They then showed that chromatinized plasmid DNA can also be degraded in trans in vitro, prompting them to test whether this could be harnessed for programmed chromatin shredding. This proved to be a feasible approach for both heterologous and endogenously expressed genes, where high target abundance correlated with increased rates of cell death in several mammalian cell lines, further supported by the upregulation of multiple DNA damage markers.

To further demonstrate the therapeutic potential of SuCas12a2, the authors showed that Cas12a2-mediated transcript targeting enables selective killing of cancer cells, including those with oncogene deletions or TP53 point mutations. Using the PC9 human NSCLC cell line, they demonstrated robust in vitro killing by delivering SuCas12a2 with LNPs. Following this, in vivo experiments in mice revealed that LNP-delivered SuCas12a2 RNPs achieved modest tumor suppression and reduced metastasis.

Overall, this is a data-rich study in which the authors explore multiple contexts to demonstrate the efficacy of Cas12a2 in eliminating specific cells. The study will be of particular interest to audiences focused on cancer therapeutics and novel CRISPR-based approaches. However, in some instances (see below), the descriptions of results are not fully supported by the data presented. Another weakness is that the applicability of the tool, as currently described, lacks sufficient foundation to be considered a reliable therapeutic strategy. For example, conclusive guidelines for selecting a gRNA and/or target transcript are largely missing and remain to be established. Additionally, it is unclear whether the differentiating power of the tool arises from an optimal PFS or is protospacer-dependent. As detailed below, incorporation of the suggested changes could make this study a good candidate for readership of Nature.

We thank the reviewer for the careful and constructive evaluation of our manuscript. We appreciate the reviewer's recognition that "the study will be of particular interest to audiences focused on cancer therapeutics and novel CRISPR-based approaches," as well as the thoughtful suggestions for strengthening the mechanistic framework and applicability of the approach. In particular, we agree that more rigorous characterization of gRNA design principles is important for establishing Cas12a2 as a practical therapeutic platform. In response to these

comments, we have expanded the analysis of single-nucleotide discrimination, clarified the respective roles of PFS context and protospacer mismatches in guide design, and revised several sections of the manuscript to ensure that the interpretations more accurately reflect the data and the current limitations of the approach. We address each point below.

Major comments

The study could benefit from a more thorough and systematic approach towards characterizing the applicability limits of Cas12a2 before using it as a therapeutic tool. At various points in the manuscript, the gRNA designs appears to follow a hit-or-miss strategy. For example, in Figure 4, the authors make use of a single-nucleotide difference in the PFS for the Cas12a2-mediated selective elimination of cells expressing the mutant (R248Q) p53 gene. This is somewhat surprising, as the PFS was classified as rather flexible by the Dmytrenko et al. 2023 study. The authors should be more clear about whether their findings (also for other guide RNAs tested) are consistent with the proposed tolerated PFS motifs (or protospacer mismatch positions) from the Dmytrenko et al study and more importantly, how much this limits the applicability of Cas12a2 to differentiate between target RNAs with a single-nucleotide resolution.

Another example can be found in Extended Data Fig. 6e, where six different gRNAs are tested for specific targeting of a mutant p53, but the rationale for their design and evaluation of their performance are not clearly explained. The targeting efficiencies vary, and it is unclear what conclusions can be drawn from these results. For example, most guide RNA appear to be non-functional, g4 shows trans cleavage activity on both WT and mutant targets (but this is not addressed in the text), while g6 appears to perform best. Also here, it would be insightful if the authors could compare this with the Dmytrenko et al. study (e.g. were PFS distal mutations more effective?).

We thank the reviewer for this important comment. In Dmytrenko *et al.* (2023), the PFS appeared relatively flexible in bacterial systems, although at least one adenine was typically present within the middle three nucleotides of the 5'-NNNNN-3' PFS motif (Extended Data Fig. 5d in Dmytrenko *et al.*). Because these experiments were performed in bacteria, the effective targeting rules may differ in mammalian cells, where intracellular conditions such as Mg^{2+} concentration may influence Cas12a2 activation. Consistent with this possibility, we observe that Cas12a2 activity is sensitive to Mg^{2+} concentration (Extended Data Fig. 1f). In addition, RNA secondary structure can influence target accessibility, and fully double-stranded RNA regions do not efficiently activate Cas12a2, as also noted in Dmytrenko *et al.*

To more systematically characterize mutation-targeting behavior in human cells, we performed a mismatch-tiling analysis at two independent TP53 loci (R248Q and R280K) (see response to Reviewer #2 comment 1). For each locus, 28 gRNAs were tiled across the mutation site, enabling evaluation of guides that recognize the mutation through the PFS as well as through protospacer mismatches (Extended Data Fig. 4). The hit gRNAs all show selectivity towards target cells (Fig.4, Extended Data Fig. 4g-h and 6a-b), demonstrating that single-nucleotide mutation discrimination can occur through spacer mismatches in addition to PFS-dependent targeting.

Interestingly, several active guides identified in this analysis do not contain the consensus 5'-GAAAG-3' PFS motif, although they all include at least one adenine within the PFS region, consistent with Dmytrenko *et al.* Conversely, multiple guides containing adenines within the PFS were inactive (e.g., p53 R248Q gRNAs 1, 2, and 26–28; Extended Data Fig. 4), indicating that PFS composition alone is not sufficient to predict guide activity in mammalian cells.

We also clarified the interpretation of data in Extended Data Fig. 6d. In the revised manuscript, we note that gRNA6 shows the strongest mutation-selective activity. To avoid confusion, we have removed gRNA4 from the sentence describing selective targeting.

Together, these results indicate that Cas12a2 can achieve single-nucleotide discrimination through both PFS-dependent recognition and spacer mismatch sensitivity in human cells. At the same time, our data suggest that targeting rules defined in bacterial systems do not fully predict guide performance in mammalian contexts. We therefore note these points in the Discussion and state that guide RNA screens in human cells are required for establishing generalizable Cas12a2 design principles.

“Mice receiving MYC-targeting LNPs exhibited reduced tumor surface area (percentage of total liver) and decreased liver-to-body weight ratios compared to control groups (Fig. 5d; Extended Data Fig. 7b-d).” This does not seem to be supported by the data. Firstly, there are two control groups in the experiment presented in Figure 5D: PBS and a non-targeting guide RNA (“LNP NT”). Whereas the targeting guide (“LNP MYC”) was significantly different from the PBS control, it does not seem to be significantly different from the “LNP NT” control, although the statistical test appears not to be performed between these two conditions. Secondly, the “liver to body weight ratios” presented in Extended Data Figure 7C appear to be very similar across the three conditions. Also here, the statistical tests are missing to substantiate these claims.

We thank the reviewer for pointing this out and agree that proper statistical comparisons are important for interpretation. In the revised manuscript, we have included statistical analyses comparing tumor surface areas between groups treated by LNP-NT and LNP-MYC ($p=0.031$, Fig. 5d). For liver to body weight ratios, LNP-MYC treated mice showed reduced ratios but this did not reach statistical significance when compared with controls. Hence, we removed the claim from the main text that MYC-targeting LNPs reduced liver-to-body-weight ratios.

Minor comments

It is highly recommended to add a table that contains all guide RNAs (and cell lines) used in this study.

We thank the reviewer for this suggestion. We have added tables describing all guide RNAs (Supplementary Table 1), cell lines and plasmid maps (Supplementary Table 4) used in this study. Plasmids used to make the cell lines will be available on Addgene.

“SuCas12a2 RNPs also cleaved supercoiled plasmid DNA in trans following RNA targeting, whereas control SuCas12a2 RNPs containing non-targeting guide RNAs did not induce RNA or

DNA cleavage (Fig. 1b).” The non-targeting guide RNA control, nor the RNA cleavage is present in this figure.

We thank the reviewer for pointing out this error. We have corrected the text by removing the second part of the sentence.

It would improve the readability if the authors were consistent with naming the gRNA/RNP used (currently it's a mix of “GFPg RNP”, “GFP gRNA”, “GFP gRNA RNP” and “GFP g”). A similar statement can be made on the inconsistent use of the Cas12 ribonucleoprotein (for example figure 1b: “Cas12a2 RNP + target RNA”, figure 1c: “Cas12a2 gRNA target RNA”, figure 1d: “Cas12a2-gRNA”, figure 2a: “Cas12a2-gRNA RNP”).

We thank the reviewer for this helpful suggestion. In the revised manuscript, we have standardized the naming for guide RNAs and ribonucleoprotein complexes throughout the text and figures. Guide RNAs are now consistently referred to as “[target] gRNA”, and ribonucleoprotein complexes as “[target] gRNA RNP.” We also standardized labeling across all figures. These changes improve clarity and consistency throughout the manuscript.

Line 84: Chromatinized plasmid appears as quite a novel term without appropriate explanations (is this a good model for chromatin) or references.

We changed the term to “plasmid assembled into chromatin” and now provide references that use similar substrates.

Line 121: Consider condensing the result section “Cas12a2 activity depends on Mg²⁺ concentration”

We thank the reviewer for this suggestion. We have shortened the section to improve clarity and conciseness.

Line 149: “Transfecting gRNAs targeting ACTB (encoding β -ACTIN) or GAPDH transcripts led to cell growth arrest and expression of DNA damage markers (Extended Data Fig. 2a, b)” The authors should indicate which ACTB gRNA was used for Extended Data Fig. 2b. Did the authors see a similar trend in the upregulation of these DNA damage markers, upon using different ‘strengths’ of ACTB gRNAs? Why did targeting the transcript of the house-keeping gene GAPDH result in almost full confluence similar to the non-target control?

We thank the reviewer for these helpful suggestions. We have now clarified that the ACTB gRNA used in Extended Data Fig. 2b is ACTB gRNA7. In addition, we performed additional immunoblotting experiments using the ACTB gRNAs analyzing DNA damage marker expression. The results show a consistent relationship between guide strength and DNA damage signaling: guides that induced stronger growth inhibition in live-cell imaging also produced higher levels of pKAP1 and γ H2AX. These results are now included in Extended Data Fig. 2d. We have removed the GAPDH gRNA result for clarity of this section.

Line 153: “GFP expression increased within 4 hours after transfecting ACTB gRNAs, peaking at around 24-48 h (Fig. 2f)”. The plateau is reached around 24h; it is unclear why the authors are

mentioning such as wide range, as the x-axis ends at 36h. Was the p21-GFP signal declining after 48h?

We thank the reviewer for pointing out this inconsistency. We have revised the text to indicate that GFP expression plateaued between approximately 24 and 36 hours, consistent with the time range shown in Fig. 2f.

Line 159: "For example, CCNE1 and MYC are frequently amplified in various cancers." The provided reference does not mention MYC. Please provide an appropriate reference.

We have now added an appropriate reference documenting MYC amplification in human cancers.

Line 168: "Whereas some gRNAs (gRNA 3, 4 and 5) induced cell growth defects in both untreated cells and Dox-treated cells, gRNA 1 and 2 induced growth defects selectively in Dox-treated cells (Extended Data Fig. 3e). These observations suggested that a targeting window exists to distinguish between high and low levels of the same transcript". The different lines in these graphs are very hard to distinguish/compare. It is unclear why gRNA that provoke cell death in the -Dox condition do not perform better (and sometimes appear to perform worse) in the +Dox condition.

The y-axis scales differ between the –Dox and +Dox conditions, and cyclin E overexpression (+Dox) alone reduces the baseline growth rate. To improve interpretability, we have additionally normalized the growth rates to the non-targeting (NT) control and now present these normalized data in a bar chart (Extended Data Fig. 3f). This normalization allows clearer comparison between conditions and better illustrates the targeting window observed for gRNAs 1 and 2.

For the results presented in Figure 4C, the authors do not discuss the "GFP g" results. Was this used as a positive control and are both cell lines making use of a GFP that was knocked in-frame of the (p21-encoding) CDKN1A gene, as described on page 6? It is a bit confusing, as the next section mentions that the RPE1 p53 R248Q cell line expresses mCherry rather than GFP (as the authors use it to differentiate the two cell lines in Figure 4D) and the methods do not provide any details either.

We indeed used different cell lines for these results. In the revised manuscript, we have re-organized Fig. 4 and now instead present GR50 dose-response curves for the relevant gRNAs, which provide a clearer and more quantitative assessment of guide selectivity. We believe this reorganization substantially improves the clarity and interpretability of this section. In addition, as suggested, we now provide a table listing all cell lines and reporter constructs used in this study (Supplementary Table 4).

The results presented in Extended Data Figure 4D show that the competition experiment included a positive control (i.e. a guide anticipated to kill both cell lines by targeting ACTB), but was not described in the corresponding results section, nor quantified in Figure 4E.

We revised the manuscript to provide a more concise description of the competition experiment and now describe the ACTB-targeting guide in the main text – "Treatment with R248Q gRNA3

depleted p53 R248Q cells, allowing WT cells to dominate the population, whereas ACTB gRNA7 eliminated both cells.”.

Please double-check the formatting of the error bars and their description in the legend; they are sometimes absent.

We now provide descriptions for all the error bars in the figure legends.

The authors might want to acknowledge and/or discuss the recent pre-print published by Jorik et al. on targeted LbuCas13a-mediated cell elimination?

We thank the reviewer for pointing this out. We have added discussion about Cas13 trans-cleavage in the first paragraph of the Introduction and cited relevant papers including the suggested one.

Referee #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #5 (Remarks to the Author):

This was a very easy paper to review, and I want to briefly explain why.

*First, cancer-selective therapies have been and will always be *the* holy grail to achieve precise and durable treatment with minimal side effects. To this day, this has not been realized.*

*Second, mutant p53-selective therapies have also been and will continue to be *the* biggest holy grail because >50% of *all* human cancers harbor TP53 mutations, and they are almost *the* pathognomonic genetic lesion in often recalcitrant tumor types (e.g. ovarian cancers). To this day, this has not been realized either, but *many*, *many* people have tried.*

*Third, most *direct* "mutant p53-selective" therapeutic attempts have only focused on 1-2 "hotspot" p53 mutations, thereby ignoring hundreds of p53 mutations (most of which remain poorly understood and understudied). Most of these have failed for many reasons, including "off-target" effects whereby e.g. small molecules have later been found to target other proteins instead of mutant p53 proteins themselves. While some exciting developments in this space are seeing some success (e.g. TP53-Y220C-targeting small molecules), I would strongly argue that, to this day, this particular holy grail has not been realized either.*

*Fourth, most of the *indirect* "mutant p53-selective" therapeutic attempts, whereby other factors that support the malignant properties of mutant p53 proteins or the fitness of mutant p53-expressing cancer cells in still mechanistically obscure ways, are just too toxic to implement in the clinic (e.g. HSP90 inhibitors). Thus, I would also strongly argue that, to this day, this particular holy grail has not been realized either.*

Fifth, even if all of the above advancements were successful and went into the clinic, the reality is that tumors will acquire treatment resistance. For targeted therapies that e.g. inhibit or target

specific mutant oncogenes (or, in this case, mutant tumor suppressor proteins like hotspot mutant p53 proteins), this often happens by direct mutation of the target itself, which e.g. ablates drug binding and selectivity. Historically, this has required follow up drug development campaigns to find molecules capable of targeting the now-mutant oncogenic driver (e.g. EGFR, EML4-ALK, BCR-ABL, and the list goes on), which takes years and millions of dollars, and always fail to reach the patients that needed it right at the moment that their tumors became resistant. And for the ones that now benefit from 2nd, 3rd, 4th, or nth generation molecules, the clock is already ticking, yet there hasn't been a way to proactively and prospectively target the resistant disease that will inevitably arise later. Thus, I would also strongly argue that, to this day, this particular holy grail has not been realized either. But it clearly needs an approach that is amenable to prospective design and testing, rational (re)programming and customization, and rapid manufacturing and therapeutic deployment.

*This study effectively *shreds* each of the above problems in a way that tremendously excites this Reviewer who has been working on (mutant) p53, including therapeutic restoration, since graduate school. It presents the most compelling strategy I have seen thus far, aside from another yet-unsolved holy grail: the now-possible therapeutic correction of genetic mutations, with mutant p53 alleles being high up in that list for this Reviewer. Most importantly, their strategy has virtually unlimited (re)programmable potential and multiplexability, even longitudinally speaking, as it is a *hit-and-run* strategy that exploits programmable RNA and Cas proteins in RNPs.*

As such, I firmly believe that this paper should be immediately accepted, at least in principle, after the authors incorporate more discussion (including some limitations) and, most importantly, key references that are missing, particularly those related to landmark in vivo studies (reviews should not suffice in this case for a number of key studies).

[We thank the reviewer for their perspective on the long-standing challenges of mutant p53 targeting therapies and the exciting potential of this platform. We thank the reviewer for highlighting the broader therapeutic context and for suggesting several important additions that have strengthened the manuscript. We address each point below.](#)

Below are some general comments in this direction:

*1. What I consider to be *the* key references in the field of therapeutic p53 restoration are completely missing:*

Martins, C. P., Brown-Swigart, L. & Evan, G. I. Modeling the therapeutic efficacy of p53 restoration in tumors. Cell 127, 1323–1334 (2006)

Ventura, A. et al. Restoration of p53 function leads to tumour regression in vivo. Nature 445, 661–665 (2007)

Xue, W. et al. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature 445, 656–660 (2007)

Feldser, D. M. et al. Stage-specific sensitivity to p53 restoration during lung cancer progression. Nature 468, 572–575 (2010)

Junttila, M. R. et al. Selective activation of p53-mediated tumour suppression in high-grade tumours. Nature 468, 567–571 (2010)

Christophorou, M. A. et al. Temporal dissection of p53 function in vitro and in vivo . Nature Genet. 37, 718–726 (2005)

We thank the reviewer for emphasizing the importance of these landmark studies. We have now incorporated all of the suggested references into the Introduction.

2. There is no discussion about the fact that the most common configuration of mutation of TP53 in human cancers is a point mutation (e.g. a hotspot mutant) over a segmental deletion at 17p that includes TP53 and several other genes. This means that cancer cells express only one type of mutant p53 mRNA molecule from the single TP53 mutant allele present; as such, they are one deletion away from becoming resistant to their therapeutic approach, which is worth discussing.

We thank the reviewer for raising this important point. Consistent with this possibility, we performed qRT-PCR analysis on residual tumors from LNP-p53-treated mice and observed reduced levels of the targeted p53 mutant transcript in treated tumors (Extended Data Fig. 9d). These results suggest that reduced transcript expression may represent one potential route of resistance. We have now added a paragraph in the Discussion addressing this issue and noting that tumors may escape treatment through downregulation of the mutant transcript. We further discuss how the programmability of Cas12a2-based approaches may allow multiplexed targeting strategies to address such resistance mechanisms.

3. Related to the above, some human tumors have two different (missense) mutant TP53 alleles, so targeting these would require multiplexing to hit each of the two mutant mRNAs. Otherwise, I would predict that they wouldn't be sensitive. This is not an experiment that is required, as I think their R248Q and R175H targeting specificity experiment is quite convincing. But this is worth discussing.

We agree multiplexing has the potential to improve efficacy and avoid resistance. We have added these points in the Discussion section.

4. Their statement "unintended p53 activation... duplication (ref. 14-16)". This statement is incomplete. There is a substantial body of work related to how unintended p53 activation in cells of and within the GI tract that can lead to radiation-induced gastrointestinal syndrome due to p53-regulated cell death (not apoptosis) (e.g. . It is also reasonably well-established that certain cells, particularly of the hematopoietic system, can be quite sensitive to p53 over-activation, so this is worth discussing, too (PMID: 20019247), in the context of those introductory sentences.

We agree that p53 activation can lead to hematopoietic system depletion and hair loss during cancer therapy. For GI tract, p53 seems to play an opposite role where its activation protects the GI tract from irradiation (PMID: 20019247). We have updated the sentence to include a note about p53-induced toxicities - "...non-targeted p53 activation can induce senescence and whole-genome duplication (ref. 23–25), causing significant dose-limiting toxicities (ref. 26–29)".

5. It is also worth discussing that their liver tumor modeling experiment employed overexpression of human wild type MYC (correct?). In this regard, I thought it would be interesting and important to discuss the fact that there are missense point mutations in MYC that evade p53-dependent apoptosis, which would benefit their discussion in at least two ways. First, the authors could discuss that their approach could also be used to target mutant MYC alleles that are also tumor-specific and over-expressed, bypassing any issues with potential "on-target off-targeting" activity in normal cells that may express higher-than-average levels of MYC, and second, this would be the perfect scenario where they could connect their two main proof-of-concept studies.

We have noted in Discussion that “*targeting both the mutation and overexpression of the same transcript, as seen with overexpression of MYC in certain cancer cases (ref. 55), could provide a dual layer of efficacy and selectivity*”. In addition, we indeed used overexpression of human wild-type MYC and have now clarified this in the results and methods sections.

6. Unless I missed it, I thought it was a bit of a missed opportunity to not perform molecular analysis on their various mutant-specific targeting experiments. For instance, a set of simple RNA-seq experiments would've been interesting to see if p53-related pathways are now being reinstated upon mutant p53 transcript targeting (e.g. pathways that the wild type protein typically would suppress, which are now active in the presence of the mutant, but then no longer active --> is that enough to reinstate parts of the tumor suppressive pathway that are inactive in the mutant state, but are now back to baseline or reinstated)? But, honestly, I see no immediate or urgent need for this type of experiment because the responses are impressive and the specific mechanism beyond the chromatin shredding one is not critical to make the point here.

We agree that molecular profiling could provide additional insight into the downstream consequences of mutant transcript targeting. Such experiments will be performed in a follow up study.

Overall, this manuscript is very innovative, timely, exciting, and important, and it is definitely a "Nature paper". It deserves to be published at the highest level, and soon.

Points 1-5 above are the most important ones for me.

P.S. — one other interesting application would be to selectively eliminate human (or mouse) embryonic stem cells from a therapeutic culture that are known to acquire TP53 mutations over time (e.g. see <https://www.nature.com/articles/nature22312>).

We thank the reviewer for this interesting suggestion and agree that our approach has potential for eliminating unwanted cells in therapeutic cultures.

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have done a commendable job of addressing the concerns raised in the first round of review. In particular, I appreciate the addition of the matching control for the EGFR-targeting cell-killing assay, which strengthens Figure 3, as well as the data demonstrating protospacer-targeted recognition of a single point mutation.

We thank the reviewer for the constructive comments provided during the first round of review, which helped us strengthen the manuscript. We also appreciate the reviewer's recognition of the revisions we made.

Two remaining concerns must be addressed:

1. The p53 cell-killing experiments still lack expression-level controls for two of the three targeted mutants. Because cell killing is dependent on expression levels, the authors must demonstrate that the expression levels of R280K and E285K are equivalent to the R175H control. Furthermore, they need to explicitly state what the wild-type control is. If wild-type p53 is being used as a control, its baseline expression is substantially lower than that of the targeted mutants, making it inappropriate for this comparison; in that case, I suggest removing these data. In lines 232–235, the text implies that the wild-type, R248Q, R280K, and R175H variants have comparable expression levels. However, no data are shown for R280K, and Extended Data Figure 4f indicates that wild-type expression is reduced by at least 20-fold. An expression-level control for E285K is also missing.

We have now added RT-qPCR data in Extended Data Fig. 4g showing that the expression levels of the p53 mutant constructs R175H, R248Q, R280K, and E285K are comparable in the cell lines.

2. I remain concerned about the lack of appropriate controls for the in vivo tumor-killing experiments. The authors argue that demonstrating specificity is unnecessary because the focus is on therapeutic potential; however, they must still definitively show that the observed activity is driven by the proposed mechanism of action. Given that p53 is central to this study, it seems inappropriate to conclude the experiments when comparing the treatment to the non-target control yields a p-value of 0.10 for p53.

We thank the reviewer for this important point. We agree that the non-targeting LNP comparison is an important control for interpreting the p53 lung tumor experiments. In the revised manuscript, we now explicitly state that non-targeting LNPs showed a slight but non-significant decrease in tumor signal relative to PBS-treated controls, consistent with prior work that lung-targeting LNP formulations may themselves mildly affect lung tumor growth. We further clarify that p53 R248Q-targeting LNPs produced a larger reduction in tumor signal that was statistically significant relative to PBS-treated animals.

Because the direct comparison between non-targeting and p53-targeting LNPs did not reach statistical significance, we have revised the manuscript to moderate our interpretation of these in

vivo data and to avoid overstatement regarding mechanism or selectivity in the lung setting. Specifically, we now describe these results more cautiously, including revising the text to state that p53 R248Q-targeting LNPs were "associated with delayed metastasis". In addition, throughout the manuscript we have aligned the wording more closely with the strength of the data, including replacing "eliminate" with "kill" or "limits cell growth" or "targets" where appropriate, and revising the title from "Selective Elimination of..." to "Targeting Cancer-Specific Mutations by Transcript-Activated Chromatin Shredding."

We believe these revisions more accurately reflect the current evidence for selectivity and anti-tumor activity *in vivo* in the mutant P53 model. The MYC-targeting liver tumor model supports the broader potential of Cas12a2-mediated anti-tumor activity *in vivo*. As noted in the Discussion, the more modest effects observed on the primary tumor in the lung model may reflect limitations of the current delivery approach. We suggest several possible directions for improving the therapeutic efficacy of this approach.

In addition, Referee #3 has stated to the editors that they don't think it is satisfactory to remove data/text rather than clarifying issues raised, in particular pertaining to their points:

We appreciate the editors' and Referee #3's concerns. In the revised manuscript, we have therefore restored the relevant data and revised the text accordingly.

-lack of non-targeting control data and targeting data for Fig. 1b.

We have updated the gel to include the non-targeting gRNA control. We have also restored the corresponding text to describe the non-targeting control.

-lack of explanation of why targeting the transcript of the housekeeping gene GAPDH resulted in almost full confluence, similar to the non targeting control, in Extended Data Fig. 2b.

We have restored the GAPDH results in Extended Data Fig. 2. The GAPDH gRNA produces a mild growth defect, similar to that observed with ACTB gRNA1. Only a single GAPDH-targeting gRNA was tested in our experiments, and thus we do not interpret this result to mean that targeting GAPDH is generally less potent than targeting ACTB. Rather, we believe this likely reflects guide-dependent variability in activity, and stronger GAPDH-targeting gRNAs likely exist. We now also provide the GAPDH gRNA sequence (CUAACGGCUGCCCAUUCUUCCU) in the Supplementary Information.

Referees' comments:

Referee #1 (Remarks to the Author):

I am satisfied with the revision and recommend publication.

Referee #3 (Remarks to the Author):

I'm pleased to see that the authors have restored the data and revised the text accordingly, after it had been removed from the previous (revised) version of the manuscript, more specifically:

- The non-targeting control data and targeting data (Fig. 1b)
- The (minor) effect of the GAPDH sgRNA on cell confluence (Extended Data Fig. 2b)

One last remaining issue is that in the 'Original gels' file that was part of the submission, panel 1c and panel 1d are mislabeled.

We thank the reviewer for pointing out this labeling error. We have corrected the mislabeled lanes in panels 1c and 1d of the "Original gels" file.

Referee #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.