nature portfolio

Peer Review File

Chemically-inducible CRISPR/Cas9 circuits for ultra-high dynamic range gene perturbation

Corresponding Author: Professor Benjamin Haley

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Srinivasan and colleagues developed a collection of improved CRISPR systems to overcome the limitations of exciting approaches. The authors developed an inducible and degron-destabilized Cas9 expression systems, applied degradation approaches to anti-CRISPR proteins, and developed a Branaplam-sensitive splice switch to control the genetic editing machinery. The approaches minimize unintended editing in the off-state while maximizing editing efficiency in the on-state. Overall, this is a valuable toolbox for the community for CRISPR editing experiments. However, I have several comments for the authors to address as the characterization, applications, and specificity of this study are incomplete:

Major points:

1) The dosing and timing of compounds used in this study are unclear. Demonstration of their activity in stabilizing or destabilizing the proteins are also not shown, making it difficult to understand the experimental design in the experiments and the comprehensiveness of evaluation. The authors should show stabilization/destabilization of their control modules and provide data to support the dosing that is selected for experiments in this study.

2) In Figure 2 ,3 and 5, while the authors show flow cytometry data for their target expression, editing of the genomic locus should also be shown to help verify their observations and efficiencies.

3) In Figure 4, the characterization of genomic and protein level disruption is insufficient to show PLK1 or H3-3A is altered and to what extent. The consequences of the H3-3A disruption should also be evaluated to show a phenotypic effect. Is there a biological observation the authors can now achieve that was not evident with prior approaches? Most critically, rescue experiments are not provided to show that these effects are on-target and need to be performed with a CRISPR-resistance expression strategy. This will be necessary for the author's conclusions and is the only biological data shown in the paper.

4) The authors comment that temporal studies are feasible with these approaches. However, this is not demonstrated, nor does it seem like it would be possible with these approaches. For example, applying the Ultra-tight TET system for KO of CD81 was performed after 10 days but the rationale for this timepoint was not explained. Does it take 10 days of KO to see quantifiable loss of CD81 on the cell surface? How soon after drug treatment(s) are there measurable effects? With these kinetics, how would it be feasible to do a genomics or proteomics study to accurately define protein function?

5) What are the cell line limitations of these systems? Most of the cell lines used in this study are easy to manipulate. How efficient are these strategies in more difficult to manipulate cancer or normal cells?

6) The approaches would be valuable if they could improve utility of in vivo studies, which can be a severe limitation of existing approaches due to leakineess. The authors should comment on in vivo potential and provide proof-of-concept support that their advance would be impactful in these settings.

Minor points:

1) There is inconsistency throughout the figures where non-significance or significance calculations are noted. The authors

more clearly clarify what is significant or non-significant in each panel, either in the legends or figure itself.

2) For flow based experiments, necessary controls such as IgG or evaluations in non-CD81 lines are not shown and would be helpful for readers to evaluate their results.

Reviewer #2

(Remarks to the Author)
In their manuscript, Srinivasan, Sun, and co-authors present a system for chemically controlled expression and activity of CRISPR-Cas9 in mammalian cells. The authors initially employed the conventional reverse tetTA system for Dox-dependent Cas9 expression in standard mammalian cell lines but observed significant leakiness, i.e. Cas9 activity in the non-induced state. To address this issue, they explored various strategies, identifying the most effective approaches as (i) destabilizing Cas9 with a degradation domain and (ii) co-expressing a drug-dependent AcrIIA4 as a conditional Cas9 antagonist.

Following genomic integration of this multi-component system, the authors demonstrated strong drug-mediated control of Cas9 activity, as evidenced by antibody-based detection of a Cas9-targeted cell surface antigen via flow cytometry. They also tested an alternative system based on Branaplam-induced splicing. While generally effective, this approach exhibited strong cell-type dependence. Finally, the authors extended their strategy from catalytically active Cas9 to CRISPRi using dCas9-ZIM3, showcasing regulation of cell growth by targeting the PLK1 promoter.

Overall, this is a well-executed study and a well-written manuscript. However, I am divided on its suitability for publication in Nature Communications. While the authors successfully integrate multiple existing control strategies into a single system, the conceptual novelty remains overall limited. Furthermore, although the "ultra-tight" system has potential applications in genetic screening of essential genes and similar application scenarios, the manuscript does not convincingly demonstrate its superiority over alternative methods, such as chemically inducible split-Cas9 regulators (see extensive literature on chemical CRISPR-Cas9 control).

In my view, these limitations currently preclude publication in Nature Communications. However, if the authors can provide compelling evidence that their system offers a substantial performance advantage over the state-of-the-art in chemical CRISPR-Cas9 control and/or demonstrate a practical application, such as screening for gene essentiality in a growing organoid or a developing model organism embryo, I would be fully open to reconsidering my assessment.

Additionally, I offer the following specific comments for improvement:

1. Time-course experiment: The authors typically assess CRISPR-mediated knockdown only after 5-10 days of drug treatment, i.e. when editing is saturated. It is crucial to include a time-course experiment with samples taken at multiple time points (e.g., days 1, 2, 3, 5, and 10) to compare the genome editing kinetics of the ultra-tight CRISPR-Cas9 system against the conventional pTet CRISPR-Cas9. Given the nature of the ultra-tight system, its kinetics could be slower than the conventional pTet due to an overall diminished Cas9 activity, which could limit its utility for precisely timed gene knockouts.

2. A direct comparison between the ultra-tight system and leading strategies for chemical Cas9 control, e.g. inducible split-Cas9 regulators, would be highly informative.

3. The authors predominantly assess genome editing indirectly, i.e. through cell surface protein expression. To exclude potential artifacts, they should validate editing at the genomic level as well (e.g. via amplicon sequencing of the Cas9-targeted locus) for at least a subset of experiments, e.g. using 3–4 sgRNAs across 2–3 cell lines.

4. Including a constitutively expressed Cas9 (i.e. not Dox/drug-dependent) as a control would be important, particularly for the time-course experiment mentioned in point 1, so assess effects the drugs could have on Cas9 function/genome editing in general (i.e. irrespective of the author's regulatory modules).

Minor points to address:
* Page 3: "separated by a T2A linker" → be more precise, i.e. T2A mediates ribosome skipping, resulting in the expression of two separate polypeptides

* Last paragraph of the introduction: The statement beginning with "Together, these tools offer…" should be moderated, especially given the lack of direct comparison with state-of-the-art methods.

* Figure 2 legend title: The phrase "ultra-tight pTET system that is tight" should be revised for clarity.

Finally, while I have reservations about its suitability for Nature Communications, I acknowledge the strengths of this manuscript and the potential use of the ultra-tight system. If not accepted here, I am confident it would be well-suited for a well-recognized, more specialized journal.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors addressed several of my concerns during their revision. I appreciated the information that they provided on their rationale for dosing selection and experimental design. My suggestion would be to provide a summary of rationale for potential users, such that there is some detail then included in the manuscript or methods, to help rationalize for others that may be interested in these approaches. I also appreciated the time-course experiments that are now included.

Additionally, several major points were not addressed that would elevate the study such as biological application, rescues, in vivo applications, or clear comparisons to existing approaches (pointed out by Reviewer 2). At the very least, the authors should discuss some of these points as limitations of their study in the discussion.

Reviewer #2

(Remarks to the Author)
The authors have satisfactorily addressed my comments. Therefore, I now gladly recommend this manuscript for publication in Nature Communications. Congratulations to the authors on this excellent piece of work.

Minor comment:
On page 4: “Similar to flow cytometry results, upon induction, As before, pTET:Ultra-tight cells look longer than pTET cells to achieve maximal editing” Revise as appropriate

General responses to all reviewers:

We sincerely thank the editor and reviewers for their thorough evaluation of our manuscript and for the constructive feedback provided. The comments raised important points that prompted several additional experiments, which we believe have substantially improved the quality and rigor of the manuscript while also broadening the applicability of our new technology platforms. Notable new results now included are as follows: (i) Measurement of gene editing at the genomic level by NGS for two different cell lines and two different target loci highlighting the reliability of flow cytometry assays to assess Cas9 functionality (Fig. 2J, S3B-C, S4C, S5, S6E) (ii) Time course evaluation of editing kinetics at protein and genomic level for both the pTET:Ultra-tight and X^{on}-Cas9;AcrIIA4-FKBP systems (Fig. 2I-J, 6D, S6E), (iii) Evaluation of pTET:Ultra-tight in an iPSC line demonstrating expanded applicability in complex cell types (Fig. 3A-B, S4D-E), and (iv) Western immunoblotting experiments demonstrating stabilization/destabilization of Cas9 in pTET vs. pTET:Ultra-tight (Fig. S3A). Below we provide a point-by-point response to each of the reviewers' comments.

Point-by-point responses to each Reviewer:

Reviewer #1 (Remarks to the Author):

Srinivasan and colleagues developed a collection of improved CRISPR systems to overcome the limitations of exciting approaches. The authors developed an inducible and degron-destabilized Cas9 expression systems, applied degradation approaches to anti-CRISPR proteins, and developed a Branaplam-sensitive splice switch to control the genetic editing machinery. The approaches minimize unintended editing in the off-state while maximizing editing efficiency in the on-state. Overall, this is a valuable toolbox for the community for CRISPR editing experiments. However, I have several comments for the authors to address as the characterization, applications, and specificity of this study are incomplete:

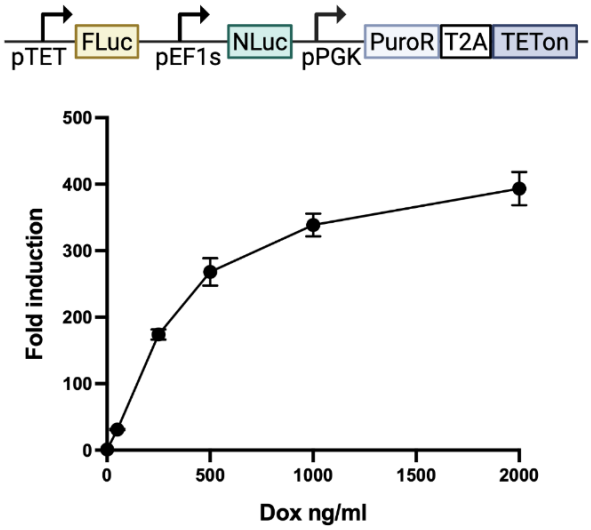
Major points:

1) The dosing and timing of compounds used in this study are unclear. Demonstration of their activity in stabilizing or destabilizing the proteins are also not shown, making it difficult to understand the experimental design in the experiments and the comprehensiveness of evaluation. The authors should show stabilization/destabilization of their control modules and provide data to support the dosing that is selected for experiments in this study.

We appreciate the reviewer's comments. In response to the question about dosing:

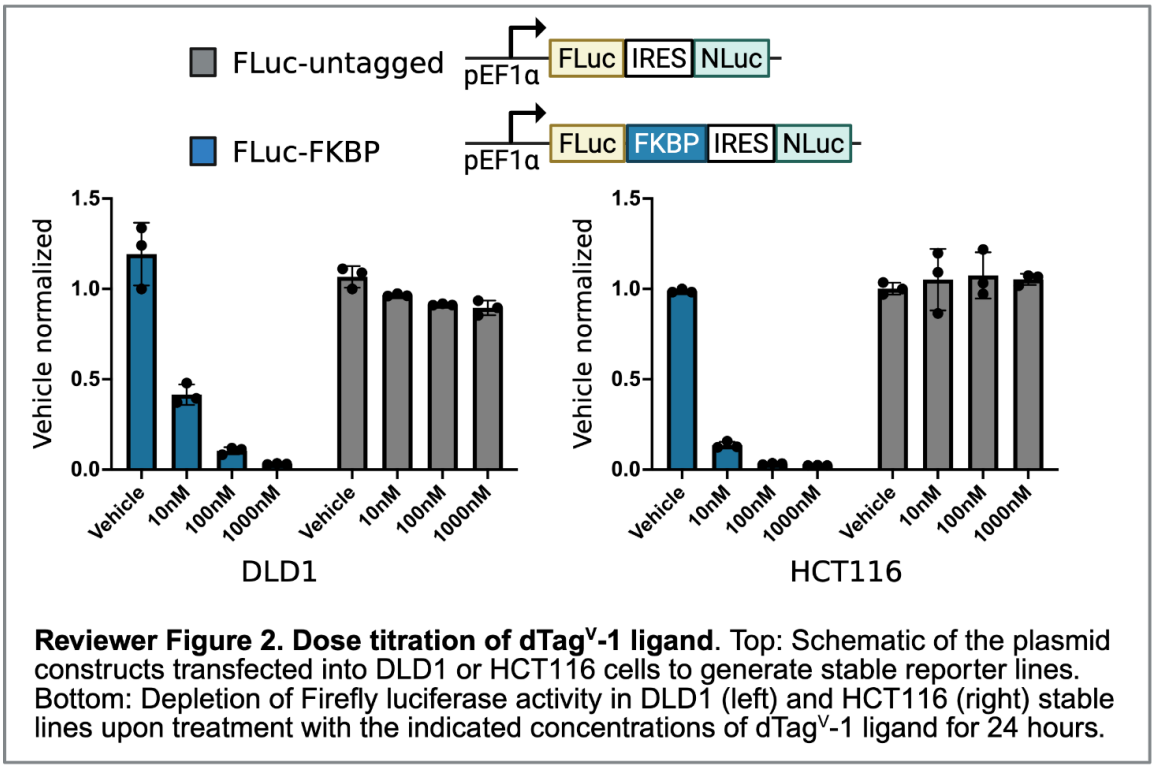
a) All of the small molecules used in this study for inducing expression or for modulating protein stability have been extensively characterized and published previously (PMID: 31101683, PMID: 31959800, PMID: 32994412, https://www.takarabio.com/learning-centers/gene-function/gene-editing/crisprcas9-delivery-methods/tet-inducible-cas9-for-gene-editing?srsltid=AfmBOoomRdgFVpxsgPgmWSfopv7wnlgm2LcYqXE5_p4UGyhPHNefkSfY, PMID: 32948771, PMID: 39504960, PMID: 21725303, PMID: 20851347, PMID: 16959577, PMID: 33795428, PMID: 34321659). In general, we referred to the published data for our dosage choice. Additionally, we performed dose titration experiments using various reporter cell lines (generated by our lab) to confirm or fine-tune published concentrations. The resulting data, combined with information from published studies, were then applied across the broader panel of cell lines used in all experiments shown for the current study. We detail our optimization process and the results in the following section.

b) To determine an optimal concentration for doxycycline (Dox), we performed a dose-response analysis using a stable, dual-reporter system in HEK293 cells (Reviewer Figure 1). In this context, **Firefly luciferase (FLuc)** is expressed from the doxycycline-inducible pTET promoter, serving as a readout for transcriptional activation, while **Nanoluciferase (NLuc)** is constitutively expressed from the EF1s promoter, serving as an internal normalization control. Cells were treated with the indicated concentrations of Dox for 48 hours, and fold induction of normalized FLuc was determined. A concentration of 2000 ng/mL Dox yielded maximal induction. This dosage aligns with concentrations used in previous studies across various cell lines (PMID: 31959800, PMID: 31101683 and PMID: 32994412) and was therefore selected for subsequent experiments.



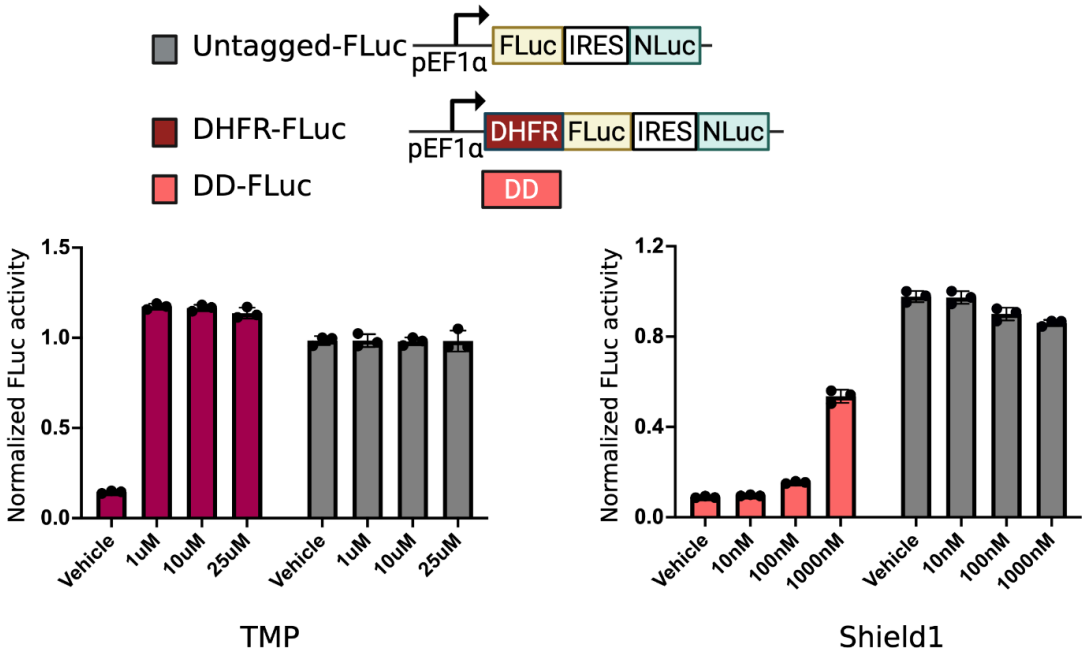
Reviewer Figure 1. Doxycycline dose titration.
Top: Plasmid schematic used to generate HEK293 stable reporter lines. Bottom: Firefly luciferase activity after 48 h treatment with indicated Doxycycline (Dox) concentrations.

To assess stabilization/destabilization via the FKBP degron tag, we generated stable DLD1 and HCT116 cell lines expressing a FLuc reporter either untagged or fused to the FKBP degron, rendering it degradable by addition of the dTAG^{V}-1 ligand (Reviewer Figure 2). NLuc was co-expressed from the same transcript via an IRES element to serve as an internal control. Cells were treated with the indicated concentrations of dTAG^{V}-1 for 24 hours, and normalized FLuc activity relative to vehicle control cells was determined. Treatment with 1000 nM dTAG^{V}-1 resulted in maximal reporter degradation in both cell lines. This concentration was consistent with doses used in prior studies across different cell types (PMID: 32948771, PMID: 39504960, PMID: 33795428) and was therefore selected for downstream experiments. FLuc activity in untagged cells remained relatively stable at all concentrations.



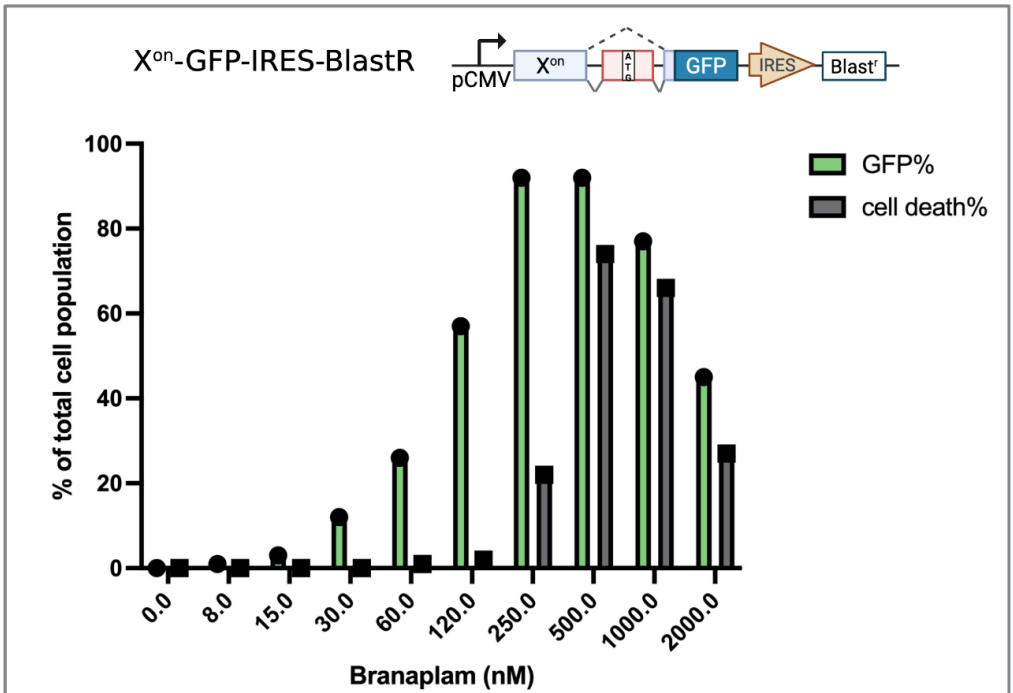
Reviewer Figure 2. Dose titration of dTag^{V}-1 ligand. Top: Schematic of the plasmid constructs transfected into DLD1 or HCT116 cells to generate stable reporter lines. Bottom: Depletion of Firefly luciferase activity in DLD1 (left) and HCT116 (right) stable lines upon treatment with the indicated concentrations of dTag^{V}-1 ligand for 24 hours.

To evaluate the functionality of destabilizing domains (DD and DHFR), we generated stable DLD1 cell lines expressing FLuc fused to either the DD or DHFR tag, resulting in constitutive degradation under basal conditions (Reviewer Figure 3). Untagged FLuc constructs were included as controls. FLuc was co-expressed with NLuc via an IRES element to enable internal normalization. Cells were treated for 24 hours with the indicated concentrations of TMP or Shield1, and normalized FLuc activity was determined relative to untagged vehicle-treated control samples. Ligand addition resulted in dose-dependent stabilization of the tagged reporters as evident from increasing FLuc activity. Based on these results 2 μM TMP and 1000 nM Shield1 were selected for use in subsequent experiments, and these doses were within the concentration range reported in previous publications in various cell lines (PMID: 21725303, PMID: 20851347, PMID: 1695957).



Reviewer Figure 3. Dose titration of TMP and Shield1 stabilizing ligands. Top: Schematic of the plasmid constructs transfected into DLD1 cells to generate stable reporter lines. Bottom: Stabilization of DHFR-tagged FLuc activity with TMP (left) and DD-tagged FLuc with Shield1 (right) in stable reporter lines upon treatment with the indicated concentrations of ligands for 24 hours. FLuc activity was normalized to NLuc and untagged vehicle control.

In order to determine appropriate Branaplam concentration for inducibility assays, stable K562 cells were generated where a GFP reporter was placed under control of the X^{on} splice switch (Reviewer Figure 4). A dosage titration of Branaplam was performed in the K562 cell line. GFP induction and cell viability (via staining of 7-AAD, Thermo Fisher Scientific) were analyzed by flow cytometry. A range of 120-250nM of Branaplam demonstrated high induction with minimal impact on cell viability. This dosage range was comparable to concentrations used in prior studies with this drug (PMID: 34321659, PMID: 35241644), and was selected for subsequent experiments in K562 cells. We selected a lower concentration (20nM) based on the previous study (PMID: 34321659), for HEK293T treatment.



Reviewer Figure 4. Dose titration of Branaplam in K562 cells. Top: Schematic of the plasmid construct transfected into K562 cells to generate stable GFP reporter lines. Bottom: Flow cytometry data displaying percentage GFP positive live cells and percentage dead cells, upon treatment with the indicated concentrations of Branaplam for 24 hours.

In summary, we referred to both previously published literature and our pilot reporter assay to select the dose of each inducer drug.

c) Please see our detailed response in point 4, which addresses a point raised by the reviewer about the timing of treatment.

d) To address the comment regarding stabilization and destabilization of the control modules, we performed immunoblotting to detect Cas9 protein in treated and untreated HEK293T cells expressing either the pTET or pTET:Ultra-tight systems (Fig. S3A). In pTET-expressing cells, Cas9 was undetectable in the absence of doxycycline but was strongly induced following treatment. Notably, untreated pTET cells still exhibited ~50% CD81 knockout by flow cytometry (Fig. 2E), despite the absence of a detectable Cas9 band. This suggests that Cas9 may have been expressed at levels below the detection threshold of our assay, or in a transient or heterogeneous manner, yet still sufficient to drive substantial genome editing. This is plausible given the potency of Cas9 when programmed with our CD81 guide RNA and the low copy number of the CD81 locus (~3 copies per cell given HEK293T is hypotriploid). These observations highlight a limitation of immunoblotting in this context: low-level Cas9 expression may evade detection while remaining functionally significant. A functional assay that measures

depletion of the target protein, as we demonstrated in the manuscript, would therefore be a more stringent approach.

For the pTET:Ultra-tight system, we analyzed Cas9 expression across untreated, single-drug-treated, and double-drug-treated conditions. As expected, Cas9 was not detected in untreated cells due to active degradation by the DD tag. Cas9 also remained undetectable in cells treated with Shield1 alone, which may reflect partial stabilization of leaky Cas9 expression that remains below the detection threshold—similar to what was observed in untreated pTET cells. A weak Cas9 band was observed with doxycycline alone, while a stronger signal was detected with combined doxycycline and Shield1 treatment. This result is consistent with dox-induced expression of Cas9-DD and Shield1-mediated stabilization of the protein.

We also attempted to detect the anti-CRISPR protein AcrIIA4, which is regulated by the LID degron in the pTET:Ultra-tight system. Despite testing both commercially available antibodies (Abcam, Diagenode), we were unable to detect AcrIIA4 by immunoblotting. However, flow cytometry data for target depletion aligned with the expected behavior of the LID-degron-tagged or the FKBP degron-tagged AcrIIA4 in response to Shield1 or $dTag^{V}$-1 treatment, respectively (Fig. 2G, 6B). Specifically, AcrIIA4 expression reduces background editing in untreated cells but also limits the extent of CD81 knockout. Addition of the appropriate degron ligand restores maximal editing, consistent with the module functioning as designed.

2) In Figure 2 ,3 and 5, while the authors show flow cytometry data for their target expression, editing of the genomic locus should also be shown to help verify their observations and efficiencies.

To address the reviewer's comment, we performed amplicon sequencing by NGS for two different targets (*CD81* and *B2M; Fig. 2J. S3C and S5)*, before and after induction of Cas9 activity, in two different cell lines (HEK293T and HAP1; Fig. 2J, S3C and S4C). We found that the KO efficiency as measured by NGS/indel rate mirrored KO efficiency measured by flow cytometry. In addition, we also performed targeted genomic sequencing as part of the newly-included time course experiments for pTET, pTET:Ultra-tight, X^{on}-Cas9, X^{on}-Cas9;AcrIIA4-FKBP (Fig. 2J, S6E). We observed strong agreement in the kinetic profiles between the protein-level (via flow cytometry) and indel generation dynamics. Interestingly, we found that indel formation preceded detectable loss of CD81 protein. This corresponds to the expected delay between genomic disruption and mRNA/protein turnover. Collectively, these new results support using flow cytometry as a quantifiable and robust readout for editing efficiency.

3) In Figure 4, the characterization of genomic and protein level disruption is insufficient to show PLK1 or H3-3A is altered and to what extent. The consequences of the H3-3A disruption should also be evaluated to show a phenotypic effect. Is there a biological observation the authors can now achieve that was not evident with prior approaches? Most critically, rescue experiments are not provided to show that these effects are on-target and need to be performed with a CRISPR-resistance expression strategy. This will be necessary for the author's conclusions and is the only biological data shown in the paper.

We thank the reviewer for this thoughtful comment. The experiments presented in Figure 4 were designed to evaluate the performance of the ultra-tight inducible CRISPR system in scenarios where conventional systems are limited by leaky effector expression—specifically, in the context of essential gene targeting (*PLK1*) and multiplexed guide dropout strategies (*H3-3A*). Our aim was to demonstrate the system's capacity to prevent premature selection and maintain balanced multi-guide activity, rather than to characterize the downstream biological consequences of gene disruption.

We agree that comprehensive phenotypic analyses and rescue experiments are critical for establishing gene-specific mechanisms. However, such studies are not required to support the conclusions of this work, which focus on the development and validation of an improved inducible genome editing platform. The observed editing at both the genomic and protein levels, in conjunction with the functional outcomes shown, sufficiently illustrate the system's utility for enabling these types of experiments.

While we appreciate the reviewer's interest in further biological investigation, we respectfully consider this to be beyond the scope of the current study, which is centered on methodological advancement. We believe the presented data provide a robust demonstration of the system's intended function and its potential to support future mechanistic studies where temporal control is essential.

4) The authors comment that temporal studies are feasible with these approaches. However, this is not demonstrated, nor does it seem like it would be possible with these approaches. For example, applying the Ultra-tight TET system for KO of CD81 was performed after 10 days but the rationale for this timepoint was not explained. Does it take 10 days of KO to see quantifiable loss of CD81 on the cell surface? How soon after drug treatment(s) are there measurable effects? With these kinetics, how would it be feasible to do a genomics or proteomics study to accurately define protein function?

We thank the reviewer for raising this important point. Our initial rationale for using a 10-day endpoint was to ensure maximal gene editing and protein depletion, while also capturing the cumulative effects of any leaky Cas9 expression; particularly relevant

when comparing inducible systems. This rationale has now been clarified in the revised manuscript.

In response to the reviewer's comment, we performed more extensive time-course experiments to assess the kinetics of CD81 disruption using the pTET, pTET:Ultra-tight, X^{on}-Cas9, and X^{on}-Cas9;AcrIIA4-FKBP systems (Fig. 2I–J, 6D, S6E). With the pTET system, ~50% CD81 loss was detected throughout the time course, consistent with substantial basal Cas9 activity. By contrast, CD81 knockout in the pTET:Ultra-tight system was only observed after inducing expression of and stabilizing Cas9, with minimal editing at early time points and progressive loss of CD81 between 24 and 72 hours. With this vector system and target gene, editing and protein loss plateaued by day 6.

Amplicon sequencing confirmed that indel formation preceded protein depletion, as expected due to mRNA/protein turnover kinetics. Importantly, our NGS results correlated well with the flow cytometry data, supporting the reliability and consistency between both genomic and protein-level readouts (Fig. 2J, S6E).

In the X^{on}-Cas9 systems, editing began within the first few days and increased through day 10. In this context, the AcrIIA4-FKBP variant demonstrated substantially reduced background editing albeit with slower activation kinetics, but still reached near-maximal editing by the final timepoint (Fig. 6D, S6E).

To summarize, while 10 days of treatment ensures complete (or near complete) editing and may be suitable for endpoint 'omics experiments, earlier timepoints (e.g., 48–72 hours) were sufficient to observe strong target protein depletion in many contexts. The optimal duration will depend on the target gene, protein half-life, and experimental objectives. Notably, the pTET:Ultra-tight system enabled controlled gene disruption with near-absent background editing, making it well suited for applications that require precise and exquisitely regulated temporal resolution. We are appreciative of the reviewer for encouraging this line of experimentation, since the ability to measure loss-of-function phenotypes before a day 10 time point would open up broader use cases for our technology platform.

5) What are the cell line limitations of these systems? Most of the cell lines used in this study are easy to manipulate. How efficient are these strategies in more difficult to manipulate cancer or normal cells?

We appreciate the reviewer's question and the broader comment regarding the importance of testing these systems in more technically demanding cell types. However, we respectfully note that the ease or difficulty of manipulating a given cell line is highly

context-dependent. What is routine in one lab may be considerably more challenging in another, depending on available protocols, infrastructure, and expertise.

To more directly address this concern, we evaluated both pTET and pTET:Ultra-tight in the KOLF2.1J iPSC line. While increasingly used as a reference line due to its well-characterized behavior and differentiation capacity (PMID: 36459969), KOLF2.1J iPSCs are generally more sensitive to transduction and genome editing than transformed lines. In this context, pTET:Ultra-tight maintained excellent performance (in fact, it demonstrated the highest on vs. off-target editing efficiency of any cell type in our panel): background editing observed with pTET (~10%) was fully eliminated, and ~80% knockout efficiency was achieved upon induction (Fig. 3A,B). Importantly, transduction and treatment did not disrupt the undifferentiated state, as evidenced by maintained iPSC morphology and high SSEA-4 expression (Fig. S4D,E).

These results demonstrate that the pTET:Ultra-tight system offers strong temporal control and robust activity even in sensitive and difficult-to-engineer models, supporting its broader applicability across diverse research settings.

6) The approaches would be valuable if they could improve utility of in vivo studies, which can be a severe limitation of existing approaches due to leakiness. The authors should comment on in vivo potential and provide proof-of-concept support that their advance would be impactful in these settings.

We thank the reviewer for this thoughtful comment. We agree that in vivo studies could significantly benefit from the enhanced control offered by our ultra-tight systems, particularly in overcoming the challenges posed by leaky gene expression. However, conducting in vivo proof-of-concept experiments (especially in transgenic mouse models) would require extensive optimization and significant resource investment, which is beyond the scope of this current study.

That said, there is strong precedent supporting the in vivo potential of our systems. The majority of the regulatory modules incorporated into our ultra-tight pTET and X^{on}-Cas9;AcrIIA4-FKBP circuits have been successfully deployed in mouse models. For example, the Tet-ON system has been widely used in vivo and is reviewed in Godecke et al. (PMID: 19394373). Similarly, the control modules - DD/Shield1, LID/Shield1, FKBP/dTAGv1, and X^{on}/Branaplam - have all demonstrated functional efficacy in murine systems (e.g., PMID: 26757616, 18836461, 32948771, 32559430, 36097386, 34321659, and 18836461). Importantly, the pharmacological inducers, Dox and Shield1, used within the pTET:Ultra-tight system, as well as Branaplam, are bioavailable and have been shown to penetrate the blood–brain barrier (PMID: 30007162, 26757616, and 35241644).

Collectively, these provide strong rationale for the potential applicability of our ultra-tight inducible systems in vivo, and we view this as an exciting area for future investigation.

Minor points:

1) There is inconsistency throughout the figures where non-significance or significance calculations are noted. The authors more clearly clarify what is significant or non-significant in each panel, either in the legends or figure itself.

We appreciate the reviewer's attention to clarity in figure presentation. In instances where multiple groupings are shown, we focused statistical comparisons on the most relevant and informative conditions to maintain figure readability and highlight key differences. For these selected comparisons, significance has been explicitly indicated in the appropriate figure panels and legends. If the reviewer has specific panels where additional comparisons would be helpful, we would be happy to incorporate them.

2) For flow based experiments, necessary controls such as IgG or evaluations in non-CD81 lines are not shown and would be helpful for readers to evaluate their results.

We appreciate the reviewer's suggestion and added the IgG control as well as a CD81-null cell line for the flow cytometry data. The result is in Figure S1A.

Reviewer #2 (Remarks to the Author):

In their manuscript, Srinivasan, Sun, and co-authors present a system for chemically controlled expression and activity of CRISPR-Cas9 in mammalian cells. The authors initially employed the conventional reverse tetTA system for Dox-dependent Cas9 expression in standard mammalian cell lines but observed significant leakiness, i.e. Cas9 activity in the non-induced state. To address this issue, they explored various strategies, identifying the most effective approaches as (i) destabilizing Cas9 with a degradation domain and (ii) co-expressing a drug-dependent AcrIIA4 as a conditional Cas9 antagonist.

Following genomic integration of this multi-component system, the authors demonstrated strong drug-mediated control of Cas9 activity, as evidenced by antibody-based detection of a Cas9-targeted cell surface antigen via flow cytometry. They also tested an alternative system based on Branaplam-induced splicing. While generally effective, this approach exhibited strong cell-type dependence. Finally, the authors extended their strategy from catalytically active Cas9 to CRISPRi using dCas9-ZIM3, showcasing regulation of cell growth by targeting the PLK1 promoter.

Overall, this is a well-executed study and a well-written manuscript. However, I am divided on its suitability for publication in Nature Communications. While the authors successfully integrate multiple existing control strategies into a single system, the conceptual novelty remains overall limited. Furthermore, although the "ultra-tight" system has potential applications in genetic screening of essential genes and similar application scenarios, the manuscript does not convincingly demonstrate its superiority over alternative methods, such as chemically inducible split-Cas9 regulators (see extensive literature on chemical CRISPR-Cas9 control).

In my view, these limitations currently preclude publication in Nature Communications. However, if the authors can provide compelling evidence that their system offers a substantial performance advantage over the state-of-the-art in chemical CRISPR-Cas9 control and/or demonstrate a practical application, such as screening for gene essentiality in a growing organoid or a developing model organism embryo, I would be fully open to reconsidering my assessment.

We thank the reviewer for this thoughtful critique and the opportunity to clarify what we believe to be the core novelty and significance of our study. While we agree that individual regulatory components used in our system have been described previously, we respectfully submit that the design, integration, and functional optimization of these elements into a modular and highly tunable system represent a novel contribution to the field.

A key innovation lies in the incorporation of a drug-regulated anti-CRISPR protein (AcrIIA4) as a repressive module that works in parallel with inducible Cas9 expression. While anti-CRISPRs (AcrIIA4) themselves are not new, their use as conditionally regulated antagonists within a multi-tiered control system (with separate, tunable expression from Cas9) is, to our knowledge, unique. This unique module is essential for achieving both undetectable background nuclease activity and robust induced editing, and provides advantages over approaches that rely solely on inducible Cas9 expression/stabilization.

We also designed the system to exploit the opposing effects of Shield-1 on two regulatory domains (DD and LID), enabling simultaneous stabilization of Cas9 and destabilization of AcrIIA4. This coordinated use of a single small molecule to exert dual, opposing control over key effectors adds another layer of functionality that has not been demonstrated in other Cas9 regulatory systems.

Our experiments further show that not all combinations of known components yield desirable outcomes. For example, pairing the Branaplam-inducible X^{on} system with

degradation domains resulted in either minimal improvement or substantial leakiness, and combinations of the X^{on} module with a Tet-responsive promoter significantly reduced inducibility. These observations reinforce the point that system performance depends not only on component selection but on thoughtful architectural design, timing, and orthogonality between modules.

With respect to the reviewer's reference to split-Cas9 strategies, we agree that such systems have been successfully deployed, particularly in contexts where transgene size is limiting (e.g., AAV delivery) or in specialized applications such as photo-inducible gene editing. However, split-Cas9 approaches are less widely used for general in vitro applications (and to our knowledge are rarely used for high-throughput or screening contexts) where simplicity, high efficiency, and compatibility with standard delivery formats are essential. Our system was specifically designed with these broader, cell-based applications in mind.

In summary, the overall novelty of our system lies in the rational integration of complementary regulatory modules, Cas9, anti-CRISPR (new from our study), and dual-use degradation domains, into a unified, high-performance platform with consistent results across all tested contexts. We hope this perspective clarifies the unique contribution of our work and its relevance to a broad range of experimental and therapeutic applications.

Additionally, I offer the following specific comments for improvement:

1. Time-course experiment: The authors typically assess CRISPR-mediated knockdown only after 5-10 days of drug treatment, i.e. when editing is saturated. It is crucial to include a time-course experiment with samples taken at multiple time points (e.g., days 1, 2, 3, 5, and 10) to compare the genome editing kinetics of the ultra-tight CRISPR-Cas9 system against the conventional pTet CRISPR-Cas9. Given the nature of the ultra-tight system, its kinetics could be slower than the conventional pTet due to an overall diminished Cas9 activity, which could limit its utility for precisely timed gene knockouts.

We appreciate the reviewer's suggestion on the time-course experiment. We picked 5-10 days of treatment to ensure drug-induced Cas9 activity and target depletion are complete based on our empirical results and to avoid underestimating leaky editing at earlier time points.

We agree with the reviewer that a more detailed time course comparison across systems will benefit users of this technology. To further understand the kinetics of inducible Cas9, we performed a time course experiment to measure the rate of CD81

target editing before day 10 (6, 24, 48, 72, 144hrs) for pTET, pTET:Ultra-tight, X^{on}-Cas9, and X^{on}-Cas9;AcrIIA4-FKBP in HEK293T (Fig. 2I, 6D). In addition to flow cytometry to monitor loss of CD81 protein, we also measured the CD81 indel rate by amplicon sequencing (Fig. 2J, S6E).

The conventional pTET system achieves saturating indel levels faster than pTET:Ultra-tight (24 vs. 48hrs), however, this is due to the significant leaky editing rate of pTET. Loss of CD81 as measured by flow cytometry lags behind indel formation as expected. There is no sacrifice in editing rate for pTET:Ultra-tight. In fact, due to the reduction in leaky editing, the induced target disruption appears faster for pTET:Ultra-tight than pTET. By contrast, the X^{on}-Cas9;AcrIIA4-FKBP system had a slower overall editing rate and when compared to X^{on} (Fig. 6D, S6E).

To summarize, marginal differences in editing efficiency (indel formation and/or protein depletion) were observed between pTET and pTET:Ultra-tight. However, a substantial degree of editing was observed with pTET at the earliest measured time points, and subtraction of this population showed that pTET:Ultra-tight was perhaps more active upon induction. A plausible explanation for this is that the non-leaky pTET cells integrated the transgene into a less-expressive site, which both minimizes background and induced expression. Separately, the X^{on}-Cas9;AcrIIA4-FKBP system, despite near-absent background, did present slower kinetics compared to X^{on}. Given that the actual kinetics could vary between cell types and targets, we recommend researchers perform a similar time course experiment for their specific context.

2. A direct comparison between the ultra-tight system and leading strategies for chemical Cas9 control, e.g. inducible split-Cas9 regulators, would be highly informative.

We thank the reviewer for this comment. We agree that direct comparisons across technologies are important and we have included performance benchmarking against the most commonly-applied chemically-inducible Cas9 systems, specifically, dox-inducible and degron-modified Cas9, throughout the manuscript.

There are indeed several chemically-inducible Cas9 systems previously described including split Cas9. As noted above, split Cas9 is more conventionally used where transgene size is limiting (as in the case of AAV vector–based delivery, which we have published on previously; PMID: 36419469) or for niche applications like those requiring photo-inducible control of gene editing. That said, we were able to identify studies that use chemical-inducible control of one or both split Cas9 elements. While these approaches showed reduced background editing, the reported knockout efficiencies range from ~20% to ~60% at best, whereas we observe ~80-95% KO efficiency with our

ultra-tight systems in those same cell lines (see table below for a summary). References: PMID: 27363581, PMID: 25643054, PMID: 35615005, PMID: 32994412, PMID: 25848930, PMID: 27618190.

Study	**Strategy**	**Chemical inducer**	**Cell Line**	**Best %editing**
PMID: 27363581	Split Cas9	4OHT	HEK293	~25%
PMID: 25643054	Split Cas9	rapamycin	HEK293FT	~40%
PMID: 35615005	Dual control	Dox + Shield1	H9 ESC	~40%
PMID: 32994412	Safe harbor integration	Dox	Multiple	~60%
PMID: 25848930	Intein-Cas9	4OHT	HEK293	~15%
PMID: 27618190	ERT2-Cas9	4OHT	HEK293	~25%

Given this reviewer's interest in split Cas9, in particular, they are likely well aware of differences in overall Cas9 efficiency based on expression levels and their control mechanisms for each half of the split Cas9, amino acid position of the split, intein sequences, chemical dimerization strategies, etc. We feel this is an exciting area of research, but a direct comparison of all of the split Cas9 methods, and optimization of any one of them for use across multiple cell contexts, represents a full study on its own and is beyond the scope of this manuscript. We have updated the Discussion section to summarize the above information, including split Cas9.

3. The authors predominantly assess genome editing indirectly, i.e. through cell surface protein expression. To exclude potential artifacts, they should validate editing at the genomic level as well (e.g. via amplicon sequencing of the Cas9-targeted locus) for at least a subset of experiments, e.g. using 3–4 sgRNAs across 2–3 cell lines.

We thank the reviewer for this suggestion. Reviewer 1 raised a similar point, and we addressed this in response to their comment above. To summarize here, we performed targeted genome sequencing before and after chemical induction of Cas9 of two different targets *CD81* and *B2M* and for two different cell types HEK293T and HAP1 (Fig. 2J, S3C, S4C, S5). In both cell types as well as at both target loci, we found strong agreement between KO efficiency measured by indel rate and flow cytometry.

In addition, we now include targeted genomic sequencing results for the newly-included time course experiments with pTET, pTET:Ultra-tight, X^{on}-Cas9, X^{on}-Cas9;AcrIIA4-FKBP (Fig. 2J, S6E). We found that KO efficiency by indel rate and flow cytometry were well-aligned at the measured time points. As discussed in more detail above in response to the comment form Reviewer 1, we observed that indel formation preceded the loss of CD81 protein.

4. Including a constitutively expressed Cas9 (i.e. not Dox/drug-dependent) as a control would be important, particularly for the time-course experiment mentioned in point 1, so assess effects the drugs could have on Cas9 function/genome editing in general (i.e. irrespective of the author's regulatory modules).

To this reviewer's point, we now include an experiment with constitutively expressed Cas9 as a positive control for gene disruption. In brief, to be consistent with the rest of our designs, we generated a piggyBac construct with a CAG promoter-driven Cas9, a sgRNA targeting CD81 and a puromycin selective marker (Fig. S1A). We generated the cell line via piggyBac transposition and selected stable cells with puromycin for 7 days. 24 hours after puro removal, we observed that CD81 KO reached near 100%, which stayed the same until day 10 (Fig. S3B-C). Stable line generation and puromycin selection take several days. Given that Cas9 and sgRNA were co-expressed in the cells, it is not surprising that KO reached completion by the time the constitutive Cas9 line was established.

Minor points to address:

** Page 3: "separated by a T2A linker" → be more precise, i.e. T2A mediates ribosome skipping, resulting in the expression of two separate polypeptides*

Thank you. For clarity we have modified the text to "each separated by a T2A sequence to enable co-expression as distinct polypeptides"

** Last paragraph of the introduction: The statement beginning with "Together, these tools offer…" should be moderated, especially given the lack of direct comparison with state-of-the-art methods.*

We thank the reviewer for flagging this and have modified the text accordingly to "Together, these tools offer a marked contrast between induced and background Cas9 activity…"

** Figure 2 legend title: The phrase "ultra-tight pTET system that is tight" should be revised for clarity.*

We appreciate this and agree with the reviewer. The Figure 2 legend title has been changed to "Development of an "ultra-tight" pTET system".

Point-by-point response to reviewers:

We thank the reviewers for their thoughtful evaluations and constructive feedback. Below we provide a point-by-point response, with reviewer comments in **bold** and our responses in plain text. All textual changes in the manuscript are highlighted in the revised version.

Reviewer #1

The authors addressed several of my concerns during their revision. I appreciated the information that they provided on their rationale for dosing selection and experimental design. My suggestion would be to provide a summary of rationale for potential users, such that there is some detail then included in the manuscript or methods, to help rationalize for others that may be interested in these approaches. I also appreciated the time-course experiments that are now included.

We thank the reviewer for this feedback. In response, we have added a new **Methods subsection ("Small molecule dosing rationale")** that summarizes the inducer concentrations used throughout (Dox 2 µg/mL, Shield1 1 µM, dTAGv-1 1 µM, trimethoprim 2 µM, and Branaplam 20–150 nM depending on cell type). We explain that these doses were selected from internal titration assays to maximize induction or degradation while minimizing cytotoxicity, and that they align with previously reported ranges.

In addition, we have inserted a clarifying statement in the **Results (Fig. 2 introduction)** that references these concentrations and explicitly defines "dynamic range" as the fold difference between residual editing in the OFF state and the efficiency achieved in the ON state.

Additionally, several major points were not addressed that would elevate the study such as biological application, rescues, in vivo applications, or clear comparisons to existing approaches (pointed out by Reviewer 2). At the very least, the authors should discuss some of these points as limitations of their study in the discussion.

We agree with the reviewer that these aspects represent important and potential future extensions of the present work. In response, we have expanded the **Discussion** to include:

1. A new **comparisons section** that discusses how pTET:Ultra-tight relates to split/intein-Cas9 and ERT2-Cas9 systems, highlighting differences in editing efficiency, background activity, and compatibility with high-throughput applications.

2. A new **Supplementary Table S7** that summarizes reported efficiencies of representative split/intein Cas9 systems side-by-side with those achieved by pTET:Ultra-tight.

3. A new **Limitations and future directions paragraph** within the Discussion section, which explicitly acknowledges that:
    - We did not include phenotypic characterization or rescue experiments, which will be important for future biological studies.

- Although the modules employed (Tet/Dox, DD/Shield1, FKBP/dTAG, Xon/Branaplam) have demonstrated functionality in vivo, we did not conduct direct animal studies in this manuscript.
- While we discussed and summarized representative comparisons with split-Cas9 and related inducible systems, we did not perform head-to-head benchmarking against these platforms in our own experiments, which we view as an important future direction.

Together, these additions provide context for users of our technology, and frame these aspects as opportunities for extending the platform.

Reviewer #2

The authors have satisfactorily addressed my comments. Therefore, I now gladly recommend this manuscript for publication in Nature Communications. Congratulations to the authors on this excellent piece of work.

We are very grateful for the reviewer's positive evaluation and their recommendation for acceptance. We appreciate the recognition of the technical advances and conceptual contributions of this work.

Minor comment: On page 4: "Similar to flow cytometry results, upon induction, As before, pTET:Ultra-tight cells look longer than pTET cells to achieve maximal editing" Revise as appropriate.

We have corrected this sentence. It now reads:
"Similar to flow cytometry results, upon induction, pTET:Ultra-tight cells showed a modest delay compared to pTET cells before achieving maximal editing."

General manuscript improvements

- Added a standardized **statistics line** to all figure legends:
  "Bars show mean ± SD (n as indicated). Significance by [test] with [correction]; ns, not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001."
- Inserted **Supplementary Table S7** comparing published inducible Cas9 systems to pTET:Ultra-tight.

We believe these revisions address all outstanding reviewer comments and improve the clarity, balance, and long-term value of the manuscript.