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Peer Review File

A sensitive red/far-red photoswitch for controllable gene therapy in mouse models of metabolic diseases

Corresponding Author: Professor Haifeng Ye

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications. Mentions of the other journal have been redacted.

Parts of this Peer Review File have been redacted as indicated 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:

Reviewer #1

(Remarks to the Author)
Overall, the authors did not appreciate the former reviewer's comments and suggestions that, this reviewer believes, could enable them for improving the manuscript further: 1) confirm nuclear translocation of the constructs with red light (Fig. 1a, 3a and Ext. Data 5a; no change in the figures even though they said they omitted such a translocation in the figures); 2) overcome a weakness that the REDLIP system cannot directly target a single copy of endogenous gene targets, using construct optimization; 3) investigate non-specific AAV infection in vivo; 4) design appropriate application(s) of REDLIP system as a red-light inducible system for conducting proof of concept experiment and/or addressing important biological questions. Also, this reviewer cannot believe that any clinicians will be able to use this optogenetic tool for their diabetic patients as an alternative of insulin while the authors mentioned it in the rebuttal letter. Unfortunately, the current version of this manuscript is not suitable for this journal.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Qiao and their colleagues conducted several revision experiments to describe their development of REDLIP. This reviewer appreciated the authors' effort to address the provided concerns experimentally. However, the mechanism underlying FnBphP nuclear-translocation and the advantage of this system remain not clear and convincing as well as the proposed approach may have side effects. Particularly, the authors did not apply an important strength of optogenetic tools, spatiotemporal control. All the concerns are not fully addressed by this revision. Unfortunately, this reviewer does not believe that this revised manuscript is suitable to be published in this journal.

Major concerns:

Only 5-10% of FnBphP were translocated to the nucleus upon 660nm while the majority stayed in the cytoplasm. It does not make sense. The author mentioned, "EGFP-Gal4-FnBphP accumulated in the nucleus… can be transported into the nucleus upon red light illumination". This is not true. Additionally, it remains unclear the mechanism, how is Gal4-FnBphP translocated to the nucleus upon 660nm?

This reviewer is afraid that FUSn-mediated droplet formation in the nucleus may have serious side effects on endogenous transcription (beside RHOXF1) and genomic DNA replication and stability in mammalian cells, though it may boost accumulation of the transcription activators. It'd be great if this author could examine the effect of FUSn-mediated REDLIP on global transcription using RNA-seq and karyotype.

The authors mentioned, “Additionally, drug production can be regulated by precisely adjusting the duration and intensity of the LED illumination using a smartphone app.” It is unsure whether the wireless system can control light intensity precisely while on/off switch should work well.

The authors have not taken advantage of their new red light-controllable system in vivo still. It would be ideal if the authors should consider other reasonable applications beside TSLP overexpression from skeletal muscles. The effect of TSLP and insulin are “systemic”. What was previously mentioned is that optogenetic tools can provide spatiotemporal control as its major strength, compared to other stimulations, as the authors described the others, sonogenetics, electrogenetics and temperature. REDLIP could be applied to address important biological questions with spatiotemporal resolution rather than a kind of overexpression demonstration.

"Regarding REDLIP reproducibility, ... I have synthesized the REDLIP constructs via IDT and subcloned them into pcDNA3, following the supplementary information: Gal4DBD-FnDrBphP and LDB3-p65-HSF1.

I tested these constructs and found that the system was leaky in dark in mammalian cell lines, NIH 3T3 and HEK293T cells. For example, I found that leakiness of luciferase in dark was 8 times as much as mock (empty pcDNA3 vector) in the cells. GFP reporter was already highly expressed in dark. When I illuminated the cells using 660nm light for 10sec and 24hr, I could not see any red light-dependent induction using the reporters, GFP and luciferase, though I conducted a couple of rounds of the experiment series."

Minor concern:

According to the supplementary material, the authors used Gal4 DNA-binding domain, not the full-length Gal4. To avoid readers’ confusion, the authors should use Gal4 DNA-binding domain (DBD) in the figures.

Manuscript number: NCOMMS-24-16264-T

Point-by-point responses

We would like to thank the editor and the reviewer for their constructive comments. Using the revision plan we developed with the Editor, we have completed all of the experiments as planned. We trust you'll agree that our careful revision process has substantially improved our study's scientific rigor and implications. We present point-by-point responses to the Reviewer's comments (below). We start by listing the major experiments undertaken during our revision process.

1. Performed new animal experiments to demonstrate the practical application of our REDLIP system. As a proof of concept, the AAV-delivered Fn-REDLIP could mediate the expression of the TSLP transgene in the muscle of HFD mice under a smartphone-controlled LED patch (**new Fig. 7a-e and new Supplementary Fig. 17**).
2. Performed new experiments to confirm the nuclear translocation of the constructs of the REDLIP system under red light. Our new data show that the Gal4-FnBphP module can be transported into the nucleus upon red light illumination (**new Supplementary Fig. 2a-c**).
3. Constructed and optimized two types of Fn-REDLIP-controlled CRISPR-dCas9 systems to evaluate the induction efficiency of SEAP reporter and endogenous *RHOXF2* gene expression upon red light illumination. (**new Supplementary Fig. 7a-f, 8a-c**).

Please note that in the main text, the first round of revisions is marked in red font; the second round of revisions is marked in blue font.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Overall, the authors did not appreciate the former reviewer's comments and suggestions

that, this reviewer believes, could enable them for improving the manuscript further: 1) confirm nuclear translocation of the constructs with red light (Fig. 1a, 3a and Ext. Data 5a; no change in the figures even though they said they omitted such a translocation in the figures); 2) overcome a weakness that the REDLIP system cannot directly target a single copy of endogenous gene targets, using construct optimization; 3) investigate non-specific AAV infection in vivo; 4) design appropriate application(s) of REDLIP system as a red-light inducible system for conducting proof of concept experiment and/or addressing important biological questions. Also, this reviewer cannot believe that any clinicians will be able to use this optogenetic tool for their diabetic patients as an alternative of insulin while the authors mentioned it in the rebuttal letter. Unfortunately, the current version of this manuscript is not suitable for this journal.

Reply:

The reviewer may have some misunderstandings. We would like to declare that we appreciate the reviewer's comments. Since the manuscript was transferred from [redacted] to *Nature Communications*, we did not address the second round of review comments with new experiments.

1) confirm nuclear translocation of the constructs with red light (Fig. 1a, 3a and Ext. Data 5a; no change in the figures even though they said they omitted such a translocation in the figures);

Reply:

Thank you for your suggestions. To confirm the nuclear translocation of the constructs under red light illumination, we fused a hybrid DNA-binding protein (Gal4-FnBphP) to EGFP (P_{hCMV}-EGFP-Gal4-FnBphP-pA) and its interacting partner LDB3 to mCherry (P_{hCMV}-LDB3-mCherry-pA) (**Supplementary Fig. 2a**). Both constructs, along with the reporter plasmid pYZ430, were co-transfected into HEK-293T cells. Twenty-four hours post-transfection, one group of cells was exposed to red light (660 nm, 1.5 mW/cm^2) for 24 hours, while the control group was kept in the dark (**Supplementary Fig. 2b**). In the dark, EGFP-Gal4-FnBphP was exclusively observed in the cytoplasm. However,

upon illumination at 660 nm, EGFP-Gal4-FnBphP accumulated in the nucleus and colocalized with LDB3-mCherry (**Supplementary Fig. 2c**). This result indicates that the Gal4-FnBphP module can be transported into the nucleus upon red light illumination.

Figure Redacted

Response Document Figure 1 (see Supplementary Fig. 2 in the revised manuscript): Red light induced the nuclear translocation of the REDLIP system constructs. a, Schematic representation of the genetic configuration for the red light-inducible nuclear translocation of the REDLIP system constructs. **b**, Schematic of the time schedule and experimental procedure for studying the nuclear translocation. **c**, Confocal imaging of red light-inducible nuclear localization of the REDLIP system constructs. HEK-293T cells were co-transfected with pQL500 (P_{hCMV}-EGFP-Gal4-FnBphP-pA), pQL501 (P_{hCMV}-LDB3-mCherry-pA) and pYZ430. Twenty-four hours after transfection, one group was illuminated with red-light (660 nm, 1.5 mW/cm^2) for 24 hours, while the other group was kept in the dark. Cells were then fixed and analyzed by confocal microscopy. Scale bars: 5 μm.

2) overcome a weakness that the REDLIP system cannot directly target a single copy of endogenous gene targets, using construct optimization;

Reply:

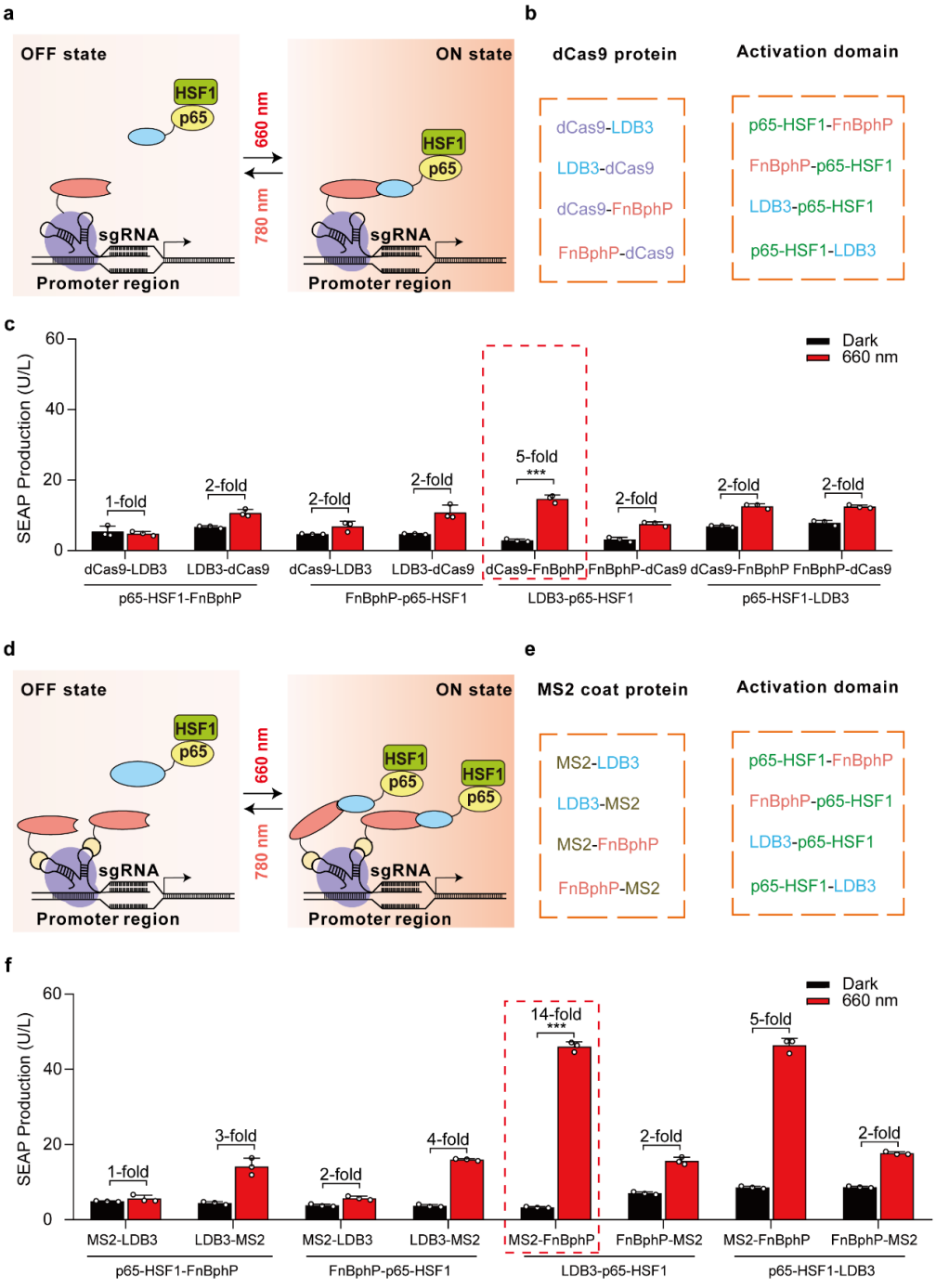
Thanks for this suggestion. We conducted new experiments to construct and optimize the REDLIP system for direct activation of endogenous genes. We designed two types of Fn-REDLIP-controlled CRISPR-dCas9 system. In the first design, the chimeric FnBphP or its interacting partner LDB3 is directly fused to either the N- or C-terminus of the dCas9 protein, creating a fusion light sensor domain (dCas9-FnBphP/-LDB3 or FnBphP-/LDB3-dCas9) driven by the human cytomegalovirus promoter (P_{hCMV}). LDB3 or FnBphP is also fused to the N- or C-terminus of the transcriptional activation domain (p65-HSF1), forming an RL-dependent *trans*-activator (LDB3-/FnBphP-p65-HSF1 or p65-HSF-LDB3/-FnBphP) driven by P_{hCMV} (**Supplementary Fig. 7a, b**). In the second design, the chimeric FnBphP or its interacting partner LDB3 is directly fused to either the N- or C-terminus of the MS2 protein, creating a fusion light sensor domain (MS2-FnBphP/-LDB3 or FnBphP-/LDB3-MS2) driven by P_{hCMV}. LDB3 or FnBphP is also fused to the N- or C-terminus of the transcriptional activation domain (p65-HSF1), forming an RL-dependent *trans*-activator (LDB3-/FnBphP-p65-HSF1 or p65-HSF-LDB3/-FnBphP) driven by P_{hCMV} (**Supplementary Fig. 7d, e**).

In the first design, we constructed all the configurations and tested their transcriptional activities using a SEAP reporter in HEK-293T cells (**Supplementary Fig. 7b**). We found that one combination (dCas9-FnBphP and LDB3-p65-HSF1) showed a 5-fold induction upon RL illumination (**Supplementary Fig. 7c**). We hypothesize that steric hindrance might hinder the binding interactions between dCas9-FnBphP and LDB3-p65-HSF, resulting in lower transcriptional activation efficiency due to challenges in the spatial conformation of the proteins involved.

To further improve the activation efficiency, we developed the second design. We constructed all configurations for the MS2 coat protein and the hybrid trans-activator, founding that one combination (MS2-FnBphP and LDB3-p65-HSF1) had the highest fold induction (14-fold induction) upon RL illumination (**Supplementary Fig. 7f**).

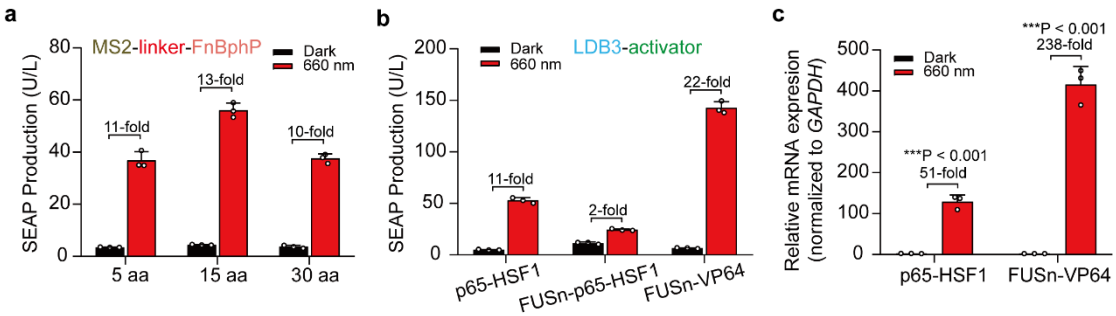
To further improve activation efficiency, we designed and compared different linkers between MS2 and FnBphP. However, there was no additional increase in SEAP reporter gene activation in response to RL illumination (**Supplementary Fig. 8a**). We

then optimized the system by fusing LDB3 with various transactivators, including p65-HSF1, FUSn [N-terminal IDR of human oncogene, which can form transcription factor droplet (*Sci Adv*. 2021. PMID: 33523844)]-p65-HSF1and FUSn-VP64. We found that LDB3 fused with FUSn-VP64 resulted in higher induction efficiency, achieving a 22-fold increase in SEAP reporter activity and a 238-fold increase in endogenous *RHOXF2* gene expression upon RL illumination (**Supplementary Fig. 8b, c**).



Response Document Figure 2 (see Supplementary Fig. 7 in the revised manuscript): Fn-

REDLIP-controlled CRISPR-dCas9 system (Fn-REDLIP$_{cas}$) for target gene activation in mammalian cells. a, Schematic design of the Fn-REDLIP$_{cas}$ system for transcriptional activation by directly fusing FnBphP or its interacting partner LDB3 to dCas9 protein. **b**, Comprehensive architectures with FnBphP or LDB3 fused to either the N- or C-terminus of the p65-HSF1 transactivation domain and the dCas9 protein. **c,** Activation of SEAP reporter gene expression in eight different combinations. HEK-293T (6×10^4) cells were co-transfected with the fusion light sensor domain (left box in b), RL-dependent *trans*-activator (right box in b), a P_{RL}-driven SEAP expression vector (pWH91) and a sgRNA targeting the exogenous *SEAP* gene (pWH189, P_{U6}-sgRNA1$_{SEAP}$-pA) at a 3:3:1:1 (w/w/w/w) ratio, followed by illumination with RL (660 nm) at 1.5 mW/cm^2 for 24 hours. SEAP production was quantified 24 hours after illumination. **d**, Schematic design of the Fn-REDLIP$_{cas}$ system for transcriptional activation by directly fusing FnBphP or its interacting partner LDB3 to the MS2 coat protein. **e**, Comprehensive architectures with FnBphP or LDB3 fused to either the N- or C-terminus of the p65-HSF1 transactivation domain and the MS2 coat protein. **f,** Activation of SEAP reporter gene expression in eight different combinations. HEK-293T (6×10^4) cells were co-transfected with the fusion light sensor domain (left box in e), RL-dependent *trans*-activator (right box in e), dCas9 expression vector (pSZ69), a P_{RL}-driven SEAP expression vector (pWH91), and a sgRNA targeting the exogenous *SEAP* gene (pWH189, P_{U6}-sgRNA1$_{SEAP}$-pA) at a 3:3:2:1:1 (w/w/w/w/w) ratio, followed by illumination with RL (660 nm) at 1.5 mW/cm^2 for 24 hours. SEAP production was quantified 24 hours after illumination. All data are represented as means ± SD; $n = 3$ independent experiments. P values in c, d were calculated using a two-tailed unpaired t-test. ***$P < 0.001$.



Response Document Figure 3 (see Supplementary Fig. 8 in the revised manuscript): Optimization of the Fn-REDLIP$_{cas}$ system for target gene activation in mammalian cells. a, Optimization of the linker length between MS2 and FnBphP. HEK-293T cells (6×10^4) were co-transfected with pQL236, pWH91, pWH189, pSZ69, and MS2-linker-FnBphP expression vectors: pQL518 (5 aa), pQL508(15 aa), or pQL517(30 aa), followed by illumination with RL (660 nm, 1.5 mW/cm^2) for 24 hours; SEAP production in the culture supernatant was quantified 24 hours after illumination. **b,** Optimization of different transcriptional activators fused to LDB3. HEK-293T cells (6×10^4) were co-transfected with pWH91, pWH189, pSZ69, pQL508, and LDB3-p65-HSF1 (pQL236), LDB3-FUSn-p65-HSF1 (pQL526), or LDB3-FUSn-VP64 (pQL527), followed by illumination with RL at 1.5 mW/cm^2 for 24 hours. SEAP production was quantified 24 hours after illumination. **c,** Activation of the endogenous gene expression. HEK-293T (6×10^4) cells were co-transfected with pSZ69, pQL508, two sgRNAs targeting the *RHOXF2* locus (pSZ105, P_{U6}-sgRNA1$_{RHOXF2}$-pA; pSZ106, P_{U6}-sgRNA2$_{RHOXF2}$-pA), and either pQL236 or pQL527, followed by illumination with RL (660 nm) at 1.5 mW/cm^2 for 24 hours. Endogenous *RHOXF2* levels were

quantified by qPCR at 24 hours after illumination. *P* values in c were calculated using a two-tailed unpaired *t*-test. ***$P < 0.001$.

3) investigate non-specific AAV infection in vivo;

Reply:

As discussed with the editor, investigating non-specific AAV infection is beyond the scope of this study. AAV vectors are widely used in clinical trials as gene therapy carriers and have been commercialized in several AAV-based therapeutics. They are considered safe and have gained broad acceptance as reliable therapeutic vehicles (*Nat Rev Genet*. 2020. PMID: 32042148; *Nat Rev Drug Discov*. 2019. PMID: 30710128; *Nat Rev Drug Discov*. 2021. PMID: 33495615). The targeted nature of AAV is not the focus of our study, so it is unnecessary to perform new experiments to investigate non-specific AAV infection in mice. Future users can opt for tissue-specific promoters, like the muscle-specific promoter we used in mouse muscle tissues.

In our manuscript, we used AAV2/9 to deliver the REDLIP system to mouse muscle tissue via intramuscular injection, using a muscle-specific promoter to avoid off-target effects (**Fig. 5, 6**). During the first round of revisions at [redacted], the reviewer requested that we test the REDLIP system in deep tissues. Therefore, we administered the system into mouse liver tissue using AAV2/8 (**Supplementary Fig. 13, 16**). This approach effectively lowered blood glucose levels in type 1 diabetes model mice and controlled an anti-obesity therapeutic protein to reduce body weight in obesity model mice. AAV2/8 has been demonstrated to efficiently target liver expression through intravenous injection, as evidenced in numerous studies (*Sci Transl Med*. 2022, PMID: 36417485; *Nat Commun*. 2022, PMID: 36064805; *Nature*. 2015, PMID: 25363772; *Science*. 2021, PMID: 34326208).

4) design appropriate application(s) of REDLIP system as a red-light inducible system for conducting proof of concept experiment and/or addressing important biological questions. Also, this reviewer cannot believe that any clinicians will be able to use this optogenetic tool for their diabetic patients as an alternative of insulin while the authors

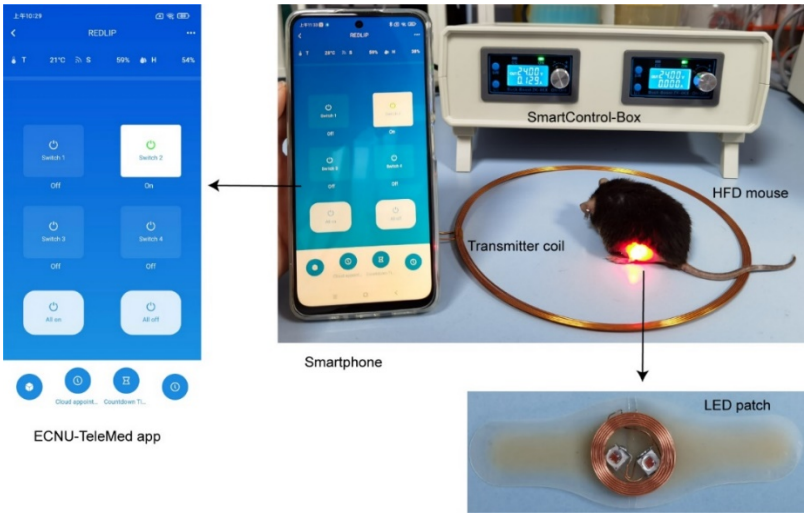
mentioned it in the rebuttal letter.

Reply:

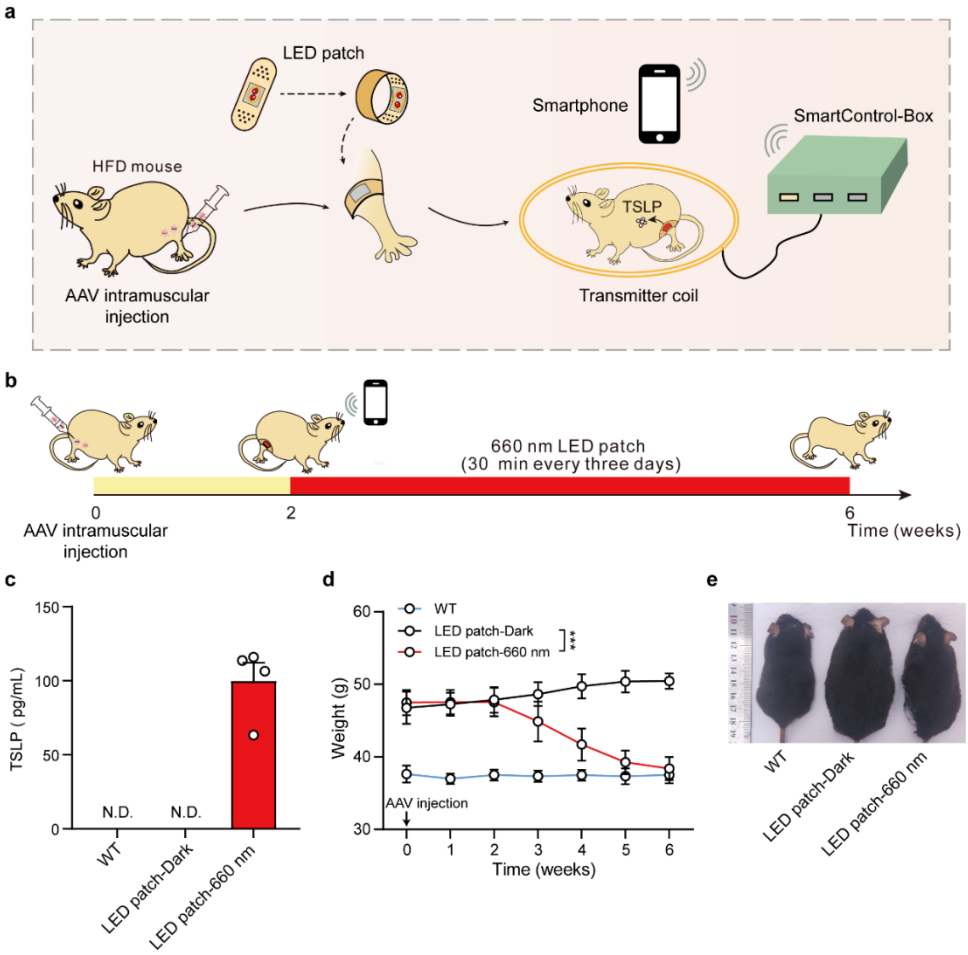
As discussed with the editor, the clinical application is outside the scope of our work, so the editorial team can set aside these concerns.

Moreover, the literature contradicts this reviewers' opinion on this matter. For example, many diabetes patients currently endure daily insulin injections, causing significant discomfort. Emerging cases utilize genetic control systems to regulate glucose homeostasis in diabetes, such as electrogenetics (*Science*. 2020, PMID: 32467389), sonogenetics (*Lancet Diabetes Endocrinol*. 2023, PMID: 37620062), and temperature sensors (*Nat Med*. 2019, PMID: 31285633). As a proof-of-concept study using optogenetic tools, we envision that future diabetes patients achieving glucose homeostasis control with short daily exposures to light. This approach aligns with our goal of developing new biotechnology tools that provide more convenient treatment options for patients.

Regarding the design and appropriate application of the REDLIP system, we believe that one of the best scenarios for its use is to modulate hormone expression on demand for therapeutic purposes. We conducted new experiments demonstrating that AAV-delivered Fn-REDLIP can mediate TSLP (thymic stromal lymphopoietin) transgene expression in the muscles of HFD mice under an LED patch controlled by a smartphone (**Supplementary Fig. 17, Fig. 7a**). HFD mice were injected intramuscularly in the gastrocnemius muscle with a mixture of AAVs encoding the Fn-REDLIP system. They were then illuminated with or without red light (RL) using an LED patch controlled by a smartphone (**Fig. 7b**), which prevents unexpected activation by ambient light. After two weeks of RL illumination, the mice in the LED patch-660 nm group showed significantly increased TSLP levels (**Fig. 7c**) and consequently reduced body weight (**Fig. 7d, e**) compared to the control mice in the LED patch-Dark group and the untreated HFD control mice.



Response Document Figure 4 (see Supplementary Fig. 17 in the revised manuscript): Experimental setup for the regulating electronic devices through a smartphone-controlled SmartController. The ECNU-TeleMed smartphone app remotely controls the SmartControl-Box via the GSM (Global System for Mobile Communications) network, automatically triggering the LED patch illumination.



Response Document Figure 5 (see Fig. 7 in the revised manuscript): AAV-delivered, Fn-REDLIP-mediated expression of TSLP in the muscles of HFD mice controlled by an LED

patch operated via smartphone. a, Schematic of smartphone controlled AAV-delivered Fn-REDLIP-mediated TSLP transgene expression in HFD mice muscles. HFD mice were injected intramuscularly in the gastrocnemius muscle with AAV encoding the Fn-REDLIP system and illuminated with or without RL (20 mW/cm^2) using the smartphone-controlled LED patch. The ECNU-TeleMed app controlled the transmitter coils through the SmartControl-Box, enabling RL-responsive Fn-REDLIP to induce TSLP expression. **b,** Schematic and timeline of the experimental procedure for AAV_{TSLP}-delivered Fn-REDLIP-mediated TSLP expression in HFD mice. HFD mice were injected with a mixture of AAV vectors pQL382 (ITR-P_{EMS}-Gal4-FnBphP-pA-ITR) and pQL383 (ITR-P_{EMS}-LDB3-p65-HSF1-pA-P_{RL}-TSLP-pA-ITR) at a titer of 2×10^{11} vg. After 2 weeks, mice were illuminated with or without RL for 30 min every three days for 6 weeks using the smartphone-controlled LED patch. **c,** TSLP levels in serum were quantified using a mouse TSLP ELISA kit. N.D., Not detected. **d,** Analysis of body weight in HFD mice treated with the AAV-delivered Fn-REDLIP system. **e,** Representative images of the LED patch-660 nm group at 6 weeks. Controls included the LED patch-Dark group, and non-model wild-type control mice (WT group). Data in **c**, **d** are presented as means ± SEM (n = 4 mice). P values in **d** were calculated by one-way ANOVA with multiple comparisons. ***$P < 0.001$.

This additional animal experiment involved placing an LED patch on the skin to illuminate the muscles and regulate the expression of an anti-obesity therapeutic protein (TSLP) for weight loss. This application serves as a proof of concept, demonstrating the potential clinical applicability of our REDLIP system. In the future, we envision that applying an LED patch on the skin could control the expression of hormone-based drugs on demand for treating diseases. Additionally, drug production can be regulated by precisely adjusting the duration and intensity of the LED illumination using a smartphone app.

We want to again express our sincere gratitude for the helpful guidance about how to improve our study and manuscript. Many thanks!

Manuscript number: NCOMMS-24-16264-A-Z

Point-by-point responses

Reviewer #1 (Remarks to the Author):

Qiao and their colleagues conducted several revision experiments to describe their development of REDLIP. This reviewer appreciated the authors' effort to address the provided concerns experimentally. However, the mechanism underlying FnBphP nuclear-translocation and the advantage of this system remain not clear and convincing as well as the proposed approach may have side effects. Particularly, the authors did not apply an important strength of optogenetic tools, spatiotemporal control. All the concerns are not fully addressed by this revision. Unfortunately, this reviewer does not believe that this revised manuscript is suitable to be published in this journal.

Major concerns:

Only 5-10% of FnBphP were translocated to the nucleus upon 660nm while the majority stayed in the cytoplasm. It does not make sense. The author mentioned, "EGFP-Gal4-FnBphP accumulated in the nucleus… can be transported into the nucleus upon red light illumination". This is not true. Additionally, it remains unclear the mechanism, how is Gal4-FnBphP translocated to the nucleus upon 660nm?

Reply:

Thank you for your feedback. We have repeated the experiments to investigate the red light-induced nuclear translocation of the REDLIP system constructs and have updated the data accordingly. We fused a hybrid DNA-binding protein (Gal4 DBD-FnBphP) to EGFP (pQL500: P_{hCMV}-EGFP-Gal4 DBD-FnBphP-pA) and its interacting partner LDB3 to mCherry (pQL501: P_{hCMV}-LDB3-mCherry-pA) (new **Supplementary Fig. 2a**). In the dark, LDB3-mCherry, which contains a nuclear localization signal (NLS), localized to the nucleus, while EGFP-Gal4 DBD-FnBphP remained in the cytoplasm. Upon illumination with 660 nm red light, EGFP-Gal4 DBD-FnBphP translocated to the nucleus and colocalized with LDB3-mCherry (new **Supplementary Fig. 2b, c**). This result indicates that FnBphP absorbs red light, transitioning from its inactive Pr state

(red light-absorbing) to its active Pfr state (far-red light-absorbing) (*J Biol Chem*. 2008. PMID: 18192276; *Nat Biotechnol*. 2022. PMID: 35697806), facilitating heterologous interaction with LDB3 and subsequent nuclear entry to modulate gene expression. Our findings clearly demonstrate successful nuclear translocation upon red light illumination, as evidenced by the overlap of green and red fluorescence, resulting in a yellow signal (shown in **Supplementary Fig. 2** and below). This confirms a robust induction of nuclear translocation following light exposure.

Moreover, two well-known optogenetic tools, the PhyB-PIF and BphP1/PpsR2 red light-regulated gene expression systems (*Nucleic Acids Res*. 2013. PMID: 23355611; *Nat Methods*. 2016. PMID: 27159085), are also designed based on the principle of heterologous protein binding and dissociation. Similar to our system, the PhyB-PIF and BphP1/PpsR2 systems have been shown to induce partial nuclear translocation of interacting proteins upon light stimulation, as demonstrated by confocal microscopy fluorescence images. This is consistent with our findings, where red light induces partial protein-protein interactions and subsequent nuclear import. All of these systems operate through a similar mechanism: photoactivation induces conformational changes in the photoreceptor protein, enabling it to bind to its corresponding partner protein, which then facilitates nuclear translocation.

We have included the updated data in the revised supplementary information (see new **Supplementary Fig. 2**). Please refer to **Page 5**, **Lines 137-145,** and below:

Figure Redacted

Response Document Figure 1 (see Supplementary Fig. 2 in the revised manuscript): Red light induces the nuclear translocation of the REDLIP system constructs. a, Schematic representation of the genetic configuration for the red light-inducible nuclear translocation of the REDLIP system constructs. **b,** Schematic of the time schedule and experimental procedure for studying nuclear translocation. **c,** Confocal imaging of red light-inducible nuclear localization of the REDLIP system constructs. HEK-293T cells were co-transfected with pQL500 (P_{hCMV}-EGFP-Gal4 DBD-FnBphP-pA), pYZ430, and pQL501 (P_{hCMV}-LDB3-mCherry-pA) or pcDNA3.1. Twenty-four hours after transfection, one group was illuminated with red light (660 nm, 1.5 mW/cm^2) for 24 hours, while the other group was kept in the dark. Cells were analyzed by confocal microscopy. Scale bars: 10 μm.

"To confirm the nuclear translocation of the photoreceptor BphP under red light, we fused a hybrid DNA-binding protein (Gal4 DBD-FnBphP) to EGFP (EGFP-Gal4 DBD-FnBphP), while its interacting partner, LDB3, was fused to mCherry (LDB3-mCherry) (**Supplementary Fig. 2a, b**).

We observed that no EGFP signals were evident in the nucleus of cells expressing only EGFP-Gal4 DBD-FnBphP, both in darkness and after red light illumination. However, a clear EGFP signal in the nucleus was detected in cells co-expressing EGFP-Gal4 DBD-FnBphP and LDB3-mCherry under red light illumination, as evidenced by the overlap of green and red fluorescence, resulting in a yellow signal (**Supplementary Fig. 2c**). These results suggest that the nuclear translocation of FnBphP is mediated by its interaction with LDB3 upon red light exposure."

This reviewer is afraid that FUSn-mediated droplet formation in the nucleus may have serious side effects on endogenous transcription (beside RHOXF1) and genomic DNA replication and stability in mammalian cells, though it may boost accumulation of the transcription activators. It'd be great if this author could examine the effect of FUSn-mediated REDLIP on global transcription using RNA-seq and karyotype.

Reply:

As we have discussed with the editor, FUSn-mediated droplet formation as a tool to promote transcriptional activation is already well established. Therefore, the editorial team can set aside concerns about the necessity of examining the global transcriptional effects of FUSn-mediated REDLIP using RNA-seq and karyotype analyses.

Previous studies have thoroughly explored the use of FUSn fusions with transcriptional activators to enhance gene expression via liquid-liquid phase separation (*Cell*, 2015, PMID: 26406374; *Science*, 2009, PMID: 19460965; *Nat Rev Mol Cell Biol*, 2017, PMID: 28225081). Recent advancements, including the fusion of FUS with TetR-VP16, have demonstrated improved transcriptional activation through phase separation (*Sci Adv*, 2021, PMID: 33523844). Furthermore, FUSn has been shown to enhance the transcriptional activation potential of dCas9-VPR/VP64 (*Protein Cell*, 2023, PMID: 36905356; *Nat Commun*, 2024, PMID: 39174527; *Nucleic Acids Res*, 2023, PMID: 37094074). These studies performed RNA-seq analyses and demonstrated significant target gene activation with minimal off-target effects (*Nat Commun*, 2024, PMID: 39174527; *Nucleic Acids Res*, 2023, PMID: 37094074).

Given this body of work, we believe it is unnecessary to repeat experiments

evaluating the effects of FUSn-mediated REDLIP on global transcription through RNA-seq or karyotype analyses. We have incorporated discussions of FUSn-mediated gene activation into the revised manuscript and added the relevant references. Please refer to **Page 8, Lines 233-240** for further details and below:

"Previous studies have explored the use of FUSn (N-terminal intrinsically disordered region of the human oncogene, which can form transcription factor droplets[46]) fusions with transcriptional activators to enhance gene activation through liquid-liquid phase separation, resulting in negligible off-target effects[47, 48]. Accordingly, we optimized the system by fusing LDB3 with various transactivators, including p65-HSF1, FUSn-p65-HSF1, and FUSn-VP64. LDB3 fused with FUSn-VP64 exhibited the highest induction efficiency, achieving a 22-fold increase in SEAP reporter activity and a 238-fold increase in endogenous *RHOXF2* gene expression upon RL illumination (**Supplementary Fig. 8b, c**)."

References:

Brangwynne CP, et al. Germline P granules are liquid droplets that localize by controlled dissolution/condensation. *Science*. **324**, 1729-1732 (2009).

Banani SF, et al. Biomolecular condensates: organizers of cellular biochemistry. *Nat Rev Mol Cell Biol*. **18**, 285-298 (2017).

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The authors mentioned, "Additionally, drug production can be regulated by precisely adjusting the duration and intensity of the LED illumination using a smartphone app."

It is unsure whether the wireless system can control light intensity precisely while on/off switch should work well.

Reply:

In our previous work, we demonstrated the use of a custom-designed home server, SmartController, programmed to process wireless signals, allowing a smartphone to regulate hormone production in optogenetically engineered cells implanted in mice using a far-red light (FRL)-responsive interface (*Sci Transl Med*. 2017. PMID: 28446682). In this system, the smartphone could remotely control both light intensity and illumination duration, effectively managing the *in vivo* production of therapeutics.

In this study, we further applied this technology by using AAV-delivered Fn-REDLIP to mediate TSLP expression in the muscles of high-fat diet (HFD) mice, controlled via an LED patch connected to a smartphone app (as also reported in *Sci Transl Med*. 2017. PMID: 28446682) (**Supplementary Fig. 17**). The SmartController system enabled the ECNU-TeleMed app to remotely adjust the light duration and intensity by regulating the voltage and current of wireless electronic devices. This capability allowed precise control over LED illumination, ensuring fine-tuning of transgene expression, by setting specific light exposure times and intensities through the app.

The authors have not taken advantage of their new red light-controllable system in vivo still. It would be ideal if the authors should consider other reasonable applications beside TSLP overexpression from skeletal muscles. The effect of TSLP and insulin are "systemic". What was previously mentioned is that optogenetic tools can provide spatiotemporal control as its major strength, compared to other stimulations, as the authors described the others, sonogenetics, electrogenetics and temperature. REDLIP could be applied to address important biological questions with spatiotemporal resolution rather than a kind of overexpression demonstration.

Reply:

As indicated by the editor in the decision letter, exploring alternative applications falls

outside the scope of our current study. Therefore, the editorial team can set aside these concerns.

In this work, our primary focus was the development of a highly sensitive red light-responsive optogenetic tool that does not require exogenous chromophore supplementation. This system allows for precise control of gene expression through red light illumination, emphasizing its ability to provide remote, traceless regulation of transgene expression—an advantage over chemically inducible systems. As a proof-of-concept, we demonstrated the effectiveness of the REDLIP system by packaging it into adeno-associated viruses (AAVs) to optogenetically control insulin expression, successfully lowering blood glucose levels in type 1 diabetes model mice. Additionally, we controlled TSLP (thymic stromal lymphopoietin) expression to reduce body weight in obesity model mice.

Regarding spatiotemporal control of gene expression, we have shown that our system enables spatially restricted transgene activation upon local illumination at the cellular level (**Fig. 2h and Supplementary Fig. 3h**). We believe this feature is well-suited for basic life science research, such as the precise control of protein interactions and signaling pathways in specific cells and tissues (*Proc Natl Acad Sci U S A*. 2020. PMID: 33318209). However, spatiotemporal control was not the main focus of this study. In future work, we plan to further explore the potential of the REDLIP system for spatiotemporal regulation of gene, protein and cellular activities.

"Regarding REDLIP reproducibility, ... I have synthesized the REDLIP constructs via IDT and subcloned them into pcDNA3, following the supplementary information: Gal4DBD-FnDrBphP and LDB3-p65-HSF1. I tested these constructs and found that the system was leaky in dark in mammalian cell lines, NIH 3T3 and HEK293T cells. For example, I found that leakiness of luciferase in dark was 8 times as much as mock (empty pcDNA3 vector) in the cells. GFP reporter was already highly expressed in dark. When I illuminated the cells using 660nm light for 10sec and 24hr, I could not see any red light-dependent induction using the reporters, GFP and luciferase, though I

conducted a couple of rounds of the experiment series."

Reply:

Thank you for highlighting this issue. It is well understood that all inducible transgene expression systems exhibit some level of background leakiness. As the reviewer noted, our system did show minimal leakage in the dark; however, this level of leakage is not significant enough to impact therapeutic outcomes when expressing drug proteins (**Figs. 5-7**). In fact, compared to other optogenetic gene expression systems, REDLIP displayed one of the lowest levels of dark-state activity (**Supplementary Fig. 4**).

Regarding reproducibility, we are confident in the robustness of the REDLIP system, as demonstrated in both our manuscript and through collaborations with other research groups. The system has consistently shown reliable performance in a variety of settings, both at the cellular level and in animal models.

We used SEAP and luciferase reporter genes to characterize the dynamics of the REDLIP system *in vitro* and *in vivo*, and the results indicated high reproducibility and stability throughout the experiments. Our collaborators have also successfully employed the system in their studies with consistent performance.

To address your specific concerns, we conducted additional experiments demonstrating red light-dependent induction using GFP and luciferase reporters. As illustrated in **Response Document Figure 2a**, transfected cells exposed to red light exhibited significantly higher luciferase and GFP expression compared to the dark control. Notably, clear red light-dependent induction was observed (**Response Document Figure 2b, c**).

Regarding the issues you encountered, we are unsure of the exact experimental conditions, including procedures, plasmid constructs, and light parameters. However, we are happy to provide our plasmids to assist with your experiments. Please note that due to the high sensitivity of the REDLIP system, it is crucial that the control group of transfected cells be strictly maintained in the dark to prevent unintended activation. Throughout the entire cellular experiment, green light should be used for illumination during procedures, rather than traditional white light, to avoid accidental activation of

the system.

We have provided detailed steps of the experiment for your reference and to facilitate replication. Please see below:

Cell transfection and illumination

HEK-293T cells were seeded into a 24-well plate at a density of 6×10^4 cells per well. The cells were cultured until they reached 70-90% confluence and were then co-transfected with a total of 375 ng of plasmids encoding the Fn-REDLIP system [pNX12, pQL326, and pQL248 or pYZ450 at a ratio of 2:2:1 (w/w/w)]. After transfection, the 24-well plate was wrapped in aluminum foil to prevent any unintended light exposure.

Six hours post-transfection, the medium was replaced with fresh Dulbecco's modified Eagle's medium (DMEM, Catalog no. 12100061, Gibco) supplemented with 10% fetal bovine serum (FBS, Catalog no. FBSSA500-S, AusGeneX) and 1% penicillin/streptomycin solution (Catalog no. ST488-1/ST488-2, Beyotime) under green light (530 nm) to prevent activation of the red light-sensitive system.

Twenty-four hours after transfection, the cells were either illuminated using a 12 ×8 LED array (660 nm, 1.5 mW/cm^2) for 10 seconds or 24 hours, or kept in the dark as a control. Fluorescence imaging and luciferase activity were analyzed 24 hours after the red-light illumination.

Fluorescence imaging

Fluorescence imaging of EGFP-expressing cells was conducted using an inverted fluorescence microscope (Olympus IX71, TH4-200, Olympus, Japan) equipped with an Olympus digital camera (Olympus DP71, Olympus, Japan). A 495/535 nm (B/G/R) excitation/emission filter set was used to capture EGFP signals. Images were acquired using a 480 nm excitation and 535 nm emission filter for EGFP detection.

Luciferase reporter assay

Luciferase activity was measured using a luciferase assay kit according to the manufacturer's instructions (Beyotime, China; Catalog no. RG005). HEK-293T cells were treated with 200 μl of cell lysis buffer and centrifuged at 13,800 × g for 5 minutes. The supernatants were collected, and 20 μl of luciferin substrate was added to 20 μl of the supernatant. The luminescence signal was detected using a Synergy H1 hybrid multi-mode microplate reader (BioTek Instruments, Inc.).

Figure Redacted

Response Document Figure 2: The Fn-REDLIP system mediates the expression of EGFP and luciferase. a, Schematic illustrating the timeline and experimental procedure for studying Fn-REDLIP-mediated EGFP and luciferase expression. **b,** Fn-REDLIP system mediates EGFP expression. HEK-293T cells were co-transfected with pQL326 (P_{hCMV}-Gal4 DBD-FnBphP-pA), pNX12 (P_{hCMV}-LDB3-p65-HSF1-pA), and pQL248 (5×UAS-P_{TATA}-EGFP-pA). Twenty-four hours post-transfection, the cells were illuminated with red light (RL, 660 nm, 1.5 mW/cm²) for the indicated times (0, 10 s, 24 h). Fluorescence imaging was performed 24 hours after the initial illumination. Scale bars: 200 μm. **c,** Fn-REDLIP system mediates luciferase expression. HEK-293T cells were co-transfected with pQL326 (P_{hCMV}-Gal4 DBD-FnBphP-pA), pNX12 (P_{hCMV}-LDB3-

p65-HSF1-pA), and pYZ450 (5×UAS-P_{TATA}-luciferase-pA). Twenty-four hours post-transfection, the cells were illuminated with RL (660 nm, 1.5 mW/cm^2) for the indicated times (0, 10 s, 24 h). Luciferase activity was measured 24 hours after the initial illumination. Data in c are presented as means ± SD; $n = 3$ independent experiments. P values were calculated using one-way ANOVA with multiple comparisons. ***$P < 0.001$.

Minor concern:

According to the supplementary material, the authors used Gal4 DNA-binding domain, not the full-length Gal4. To avoid readers' confusion, the authors should use Gal4 DNA-binding domain (DBD) in the figures.

Reply:

Thanks for your suggestion. We have made corrections according to your comments in the revised manuscript.

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Point-by-point responses

REVIEWERS' COMMENTS:

None.