nature portfolio

Peer Review File

Glucose responsive probiotics for glycemic modulation in mice and monkeys

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Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors engineer a probiotic to express GFP-1 in response to elevated glucose levels. They provide evidence for utilizing the system in mouse and NHPs with some therapeutic effects. The proof of principle seems to be demonstrated but unfortunately there are several deficiencies that severely restrict enthusiasm for this reviewer.

Major concerns
* The novelty of the manuscript is unclear. The genetic circuitry to sense glucose is a glucose responsive promoter in E. coli, similar to those that have been used in the past, e.g. Courbet STM 2015, iGEM etc. There is not sufficient characterization in confounding environments
* The authors cite the above reference 25 as “detect pathological biomarkers”, but this is misleading given it is similarly glucose sensing. The authors claim their system as “self-feedback”, which is also misleading as there is no true feedback control in the system, and the system also needs to be dosed every day
* A discussion of other efforts on engineered bacteria for treatment of diabetes is critically missing. For example, work performed in 2014 by Agarwal (DOI 10.1007/s11095-014-1430-3) using L. lactis similarly showed a constitutively expressed GLP-1 was also therapeutic, in the absence of sensing-mediated control. In Guan et al., the authors do not demonstrate the advantage over a constitutively expressed GLP, limiting the strength of claiming that such an engineered platform was required or beneficial beyond a simpler system, or its safety benefits.
* Given the inability of EcN to colonize long-term, and need for daily dosing at high levels, utility of the system is unclear. Motivation lists long-term dosing with high levels of GLP can cause adverse effects. So then, as a probiotic releasing GLP recombinantly, could also generate similar effects. It is critical to compare the author's effective concentration released by the bacteria and taken up by the subjects to establish a translational advantage to constitutive or other SOCs. What would be the concentration of direct GLP-1 delivery be, and how do dose-response effects in this or probiotic mediated systems differ
* NHP safety and efficacy data does not extend beyond 3 days. Why is this?

Minor concerns
* Unclear why Fig 1g oscillations does not translate to in vivo db/db model. It would be beneficial to observe the GLP-1 level drop in mice when the blood glucose level is low.
* Since the GIFT_GLP-1 senses the glucose in the intestine, how would the glucose from food that goes to intestine affect the sensor dynamics in vivo?
* In Supplementary Figure 7, the production of GLP-1 by GIFT is quantified from its supernatant. How is the GLP-1 released from EcN?

Referee #2

(Remarks to the Author)
In this study, Guan and colleagues developed an engineered oral glucose-sensing and self-feedback probiotic (GIFT) living drug for glucose control. They created sensor cells that temporarily reside in the intestine and regulate the expression of

GLP1 in response to glucose. The authors demonstrated a four-fold increase in plasma GLP-1 levels in db/db mice and NHPs, resulting in reduced plasma glucose levels, food intake, and body weight. The authors demonstrate increased energy expenditure, reduced levels of liver fat and inflammation, and reduced kidney complications. Based on these findings, the authors conclude that they have developed a novel method for precise therapeutic GLP1 dosing, which can be applied in the long term to promote adherence.

This work presents a new approach to treating diabetes and potentially other metabolic diseases, such as obesity and MALFD, which is of great importance. However, the reviewer has outlined several points that remain unclear and need clarification to understand the mode of action of this new approach.

Major

- The figure numbers are not in the same order as they appear in the text, which sometimes makes it difficult to follow the flow of the experiments. I would recommend that the authors streamline the figure numbers in the text with the progressive narrative of the manuscript.

- The authors demonstrate glucose-dependent sfGFP expression in cell culture through GIFT. However, the glucose concentration in the gut lumen of mice and NHPs (Figures 2, 5), where the GIFT cells temporarily reside, is not provided. This is a critical point since it is unclear whether high plasma glucose levels correspond to high intestinal glucose levels or whether glucose levels in the gut rather reflect glucose intake. For instance, can the authors demonstrate the response of a db/db mouse to a low-carb/low-calorie diet with and without GIFT in terms of GLP1, plasma glucose and insulin levels, and body weight? Does their approach also work in diet induced obese mice?

- Are the GLP1 levels in Figure 2h and 5c active or total? The authors report a four-fold increase in plasma GLP1 concentration with the administration of their GIFT cells. GLP1 receptor agonists (RA) lead to a much higher increase in total compound GLP1 levels compared to endogenous GLP1 levels. However, the effect by the GIFT cells on metabolic control seems to recapitulate most of the effects observed with GLP1-RA. Can the authors provide an explanation for this discrepancy?

- In the acute setting (Fig. 2E-J, Figures 5), GLP-1 lowers plasma glucose via stimulation of glucose induced insulin secretion. Therefore, insulin values should be presented throughout the manuscript when GIFT is applied in vivo.

- Figure 3 should include food intake data. The authors demonstrate an increase in energy expenditure that is not observed with GLP1 receptor agonists. An ANCOVA analysis should additionally be performed to analyze energy expenditure, which is the gold standard for analyzing this type of data in mice with different body weights. It would be helpful if the authors could provide an explanation for the discrepancy between their findings and the well-described effects of approved GLP1 receptor agonists on energy expenditure.

The authors state in the discussion that GIFT cells enhance insulin sensitivity in mice and NHPs. However, it is unclear whether this notion is solely based on the HOMA-IR index, which is used in humans and not validated for mice. Therefore, the authors should consider toning down this conclusion. Additionally, they should provide a more detailed explanation of what they mean by 'improved lipid metabolism'.

Finally, the authors conclude that their approach provides “a feasible and facile administration route to promote long-term adherence”. It would be helpful if they could describe the advantages over a once-weekly subcutaneous injection, considering that the GIFT cells only work temporarily and need to be replaced regularly. Additionally, it would be useful to know how this approach would work in humans.

Minor:

Abstract: The term „hypoglycemic“ agents is not correct; it should be glucose lowering agents.

Introduction: “antidiabetic drugs“ should be replaced by “glucose lowering drugs”.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
* The HexR system was also characterized in P. putida (PMID: 34508142) and I do not believe this in E. coli is a significant advance as the authors have described it.

* I did not see a proper control for a constitutive GLP production system and its effect on mice. Since the bacteria release system is different from the normal route of administration of GLP, additional characterization of the toxicity needs to be demonstrated. I don’t believe the authors have clearly demonstrated a need for a glucose-sensitive system.

* The secretion mechanism is unclear; The GLP produced by E. coli has periplasmic tags, which does not directly result in extracellular secretion and it is unclear how efficient in terms of % secretion; It is not clearly necessary in their data as many bacteria will naturally lyse and release payloads

* To evaluate primate data, the author should provide the blood glucose level of a regular monkey to compare the blood glucose level after dosing GIFT in a diabetic monkey.

* In the response document's Figure 1, how did the authors determine the concentration of the diverse carbon sources? For instance, 0.8% pyruvate (8 g/L) corresponds to approximately 90 mM, as shown in Figure 1a that they referred to, yet they opted to use 0.1 mM pyruvate for their experiment.

* The 10% bioavailability mentioned on page 16 seems unclear. The concentration of bacteria in vitro (bacterial count per culture media volume) is not equivalent to the in vivo concentration (bacterial count in the gut relative to blood volume). Therefore, bioavailability should not be calculated simply as the in vivo concentration divided by the in vitro concentration.

Referee #2

(Remarks to the Author)
The authors have responded to the questions posed in a thorough and comprehensive manner, conducting new experiments that the reviewer deems highly commendable. In their response, the authors indicate that they have adjusted the energy expenditure for lean mass. Please describe the methodology used to measure lean mass. It would be beneficial to include the lean mass, or preferably muscle mass, in the same Figure 7.
Furthermore, the authors posit that the observed increase in energy expenditure in GIFT-GLP-1-treated db/db mice is largely attributable to their increased lean body mass (p. 40/51 in the rebuttal letter). This assertion requires further substantiation. One of the most commonly observed adverse effects of GLP-1 RA and weight loss in general is a reduction in lean mass, particularly muscle mass (e.g. PMID: 39401279). If this were not the case with GIFT, it would be a significant factor in further development. The authors should therefore include the development of lean mass in their models in Figure 7, particularly in the models in comparison with semaglutide.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have conducted an array of experiments that address the major concerns raised. Key points for further strengthening would be: inclusion of an intermediate-strength constitutive promoter or mention of how the results for this may compare, and description of efficacy differences between single and multiple doses (how often does the probiotic need to be administered?). The responses from comments should be explicitly discussed in the main text and/or that the reviewer responses be published, as they contain critical details necessary for a full evaluation of the study (e.g. the promoter engineering advances, secretion details, and correct citations). Overall the work is highly innovative in its application, precedence and efficacy established in complex models.

Referee #2

(Remarks to the Author)
The authors have fully addressed my questions.

Referee #3

(Remarks to the Author)
Title: Engineered glucose-responsive probiotics for sense-and-response-based glycemic modulation in diabetic mice and monkeys
Authors: Guan et al.

Summary
This manuscript describes the engineering of an E. coli Nissle 1917 probiotic (termed GIFT) that senses intestinal glucose via a synthetic HexR-based promoter and secretes GLP-1 in a dose-dependent, reversible manner. The system achieves dynamic glycemic control in diabetic mice and non-human primates, with additional benefits on lipid metabolism, insulin sensitivity, and diabetic complications. The authors perform extensive safety profiling and benchmark the approach against semaglutide, a clinically approved GLP-1 analogue.
The work is technically rigorous, translationally relevant, and represents a significant step toward “smart” probiotic-based living medicines for metabolic disorders. The breadth of data (molecular, cellular, murine models, and NHPs) is impressive, and the concept of real-time, on-demand drug release addresses a critical unmet need in diabetes therapy.

Major Comments
1. Pharmacokinetics and Mechanism of Action

o GLP-1 exposure from GIFT is markedly lower than semaglutide (40-250 PM vs 6000-12000 PM over 6h), yet efficacy is achieved. This suggests local gut-portal signaling may dominate.
o Recommendation: Include portal vein sampling or discuss explicitly how local versus systemic GLP-1 contributes to efficacy.
2. Microbiome and Ecological Impact
o Colonization is transient, but long-term repeated dosing could alter the gut microbiome.
o Recommendation: Provide 16S rRNA profiling or at minimum discuss microbiome modulation as both a risk and potential therapeutic synergy.
3. Variability in Glucose Thresholds
o The activation threshold (~10–11 mM) is clinically relevant, but inter-individual differences in postprandial glucose kinetics may affect performance.
o Recommendation: Consider intermediate hyperglycemia models (e.g., diet-induced prediabetes) to demonstrate robustness of the sensor.
4. Adaptive Immunogenicity
o The safety analysis is comprehensive for acute responses, but adaptive immunity is not addressed.
o Recommendation: Assess antibody responses to GLP-1 or bacterial components in long-term studies.
5. Statistical Rigor and Sample Size
o Some critical endpoints (like HbA1c) rely on small cohorts (n=5).
o Recommendation: Provide power calculations or acknowledge as a limitation.

Minor Comments
• Clarify whether the final GIFT construct is plasmid-borne or chromosomally integrated. Stability will influence translational feasibility.
• Rephrase Line 83–84 (“no research has been conducted…”) to acknowledge prior probiotic engineering for metabolic regulation in animal model, while emphasizing novelty in glucose-responsive GLP-1 release in NHP and mice.
• Consider expanding the Discussion with regulatory considerations for engineered probiotics (e.g., GMP production, GMO classification).

Strengths
• Clear innovation: first oral probiotic platform enabling real-time, glucose-responsive GLP-1 release.
• Streamlined engineering of a HexR-based sensor with clinically aligned activation thresholds.
• Comprehensive validation across multiple murine models and spontaneous diabetic cynomolgus monkeys.
• Strong translational framing, including direct comparison to semaglutide.
• Extensive acute and subchronic safety profiling.

Recommendation
This is a great manuscript with novelty and broad interest, well-suited for a high-impact journal like Nature. I recommend acceptance after major revisions, addressing the mechanistic, ecological, and immunogenicity concerns outlined above.

Referee #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #5

(Remarks to the Author)
This manuscript describes the development of GIFT (Glucose-Responsive Intestinal Functional Therapeutics), an engineered probiotic system using E. coli Nissle 1917 as a chassis for glucose-responsive delivery of GLP-1. The core innovation is a synthetic glucose-sensing circuit based on the HexR transcriptional repressor from Neisseria meningitidis, which is derepressed by the glycolytic intermediate KDPG when glucose levels exceed approximately 10 mM. The authors demonstrate that this system activates gene expression preferentially in diabetic mice with elevated blood glucose while remaining quiescent in normoglycemic animals. When engineered to produce a secreted GLP-1 variant, the system reduces fasting blood glucose, improves glucose tolerance, lowers HbA1c, and ameliorates several diabetic complications in db/db mice over 30-day treatment periods. The authors extend these findings across multiple mouse models (DIO, DIO+STZ) showing that efficacy correlates with disease severity, and provide supporting data from a small non-human primate study. The work is positioned as proof-of-concept for a "microbial incretin mimic" approach to chronic glycemic management rather than real-time closed-loop glucose control.

The manuscript presents an ambitious and technically sophisticated body of work that addresses a genuine unmet need in diabetes therapeutics. The fundamental concept of glucose-responsive therapeutic delivery from an orally administered probiotic is scientifically compelling, and the authors have assembled a substantial dataset spanning in vitro characterization, multiple rodent disease models, and preliminary primate studies.

Several aspects of the work are commendable: the demonstration of differential activation between normoglycemic and hyperglycemic mice provides direct functional evidence of glucose-responsiveness in vivo; the GIFTLux control appropriately isolates GLP-1-dependent effects from chassis effects in the primary efficacy experiments; and the multi-model validation adds confidence that observations are not model-specific artifacts. The comparison to semaglutide safety profiles

raises interesting questions about the potential advantages of physiological-level versus supraphysiological GLP-1 delivery. The comprehensive safety data, including the two-month comparative study with semaglutide examining complete blood counts, serum biochemistry, and inflammatory markers, substantially strengthens the translational case. The body composition data suggesting potential preservation of lean mass with GIFTGLP-1 treatment compared to semaglutide is intriguing and could represent a meaningful clinical advantage if confirmed in further studies.

The characterization of the secretion mechanism, including SecA inhibitor experiments and quantification of secretion efficiency, is thorough. The demonstration that GLP-1 released through bacterial lysis lacks biological activity due to retention of the uncleaved signal peptide effectively addresses the question of whether active secretion versus passive lysis drives therapeutic effects. The comparison with constitutive expression systems (both strong and moderate promoters) provides valuable context for why glucose-responsive regulation is advantageous.

However, several issues require attention. The manuscript does not address the colibactin genotoxicity concern associated with the pks genomic island present in E. coli Nissle 1917—a significant omission for a therapeutic intended for chronic daily administration. Some methodological details require clarification. The NHP data, while including appropriate healthy and untreated diabetic controls, lack a chassis-only control that would definitively isolate GLP-1-specific effects in primates. Furthermore, the mechanistic argument that portal vein delivery explains therapeutic efficacy at GLP-1 concentrations roughly two orders of magnitude below those achieved by approved GLP-1 agonists, while pharmacologically plausible, remains incompletely substantiated. The points below detail these and other concerns that should be addressed.

Specific Concerns

The manuscript does not address the colibactin genotoxicity concern associated with the pks genomic island present in E. coli Nissle 1917. Multiple studies have demonstrated that EcN produces the genotoxin colibactin, which induces DNA double-strand breaks in epithelial cells and has been linked to colorectal cancer risk in mechanistic studies. While standard Ames tests may not detect this specific genotoxic mechanism, and while EcN has an extensive history of human use as a probiotic, a manuscript proposing chronic daily therapeutic administration of this chassis should at minimum discuss this controversy.

The non-human primate study includes appropriate comparisons with healthy non-diabetic monkeys and untreated diabetic monkeys, which provides meaningful context for interpreting the magnitude of therapeutic effects. However, the study lacks a concurrent GIFTLux (or other chassis-only) control group. While the within-subject pre-treatment baseline and the untreated diabetic control group provide substantial protection against confounding, and while the mouse data with GIFTLux controls strongly suggests effects are GLP-1-mediated, a parallel chassis-only control in primates would more definitively rule out that observed effects derive from chassis-related factors rather than GLP-1 specifically. Given the comprehensive GIFTLux control data in rodents, this concern is somewhat mitigated, but explicit acknowledgment of this limitation would strengthen the manuscript.

The GLP-1 ELISA methodology requires clarification regarding sample collection procedures. While the methods specify use of an active GLP-1 kit (Mercodia EGLP-35K), they do not indicate whether blood samples were collected with DPP-IV inhibitors, which is standard practice given the extremely short half-life of active GLP-1. If inhibitors were used, this should be stated; if not, the measured values may underestimate true active GLP-1 concentrations at the time of collection, which affects interpretation of the therapeutic mechanism.

The manuscript claims therapeutic efficacy at systemic GLP-1 concentrations approximately 25-300 fold lower than those achieved by semaglutide. The proposed explanation—that intestinal delivery provides favorable portal vein pharmacokinetics mimicking native incretin secretion—is physiologically reasonable but not directly demonstrated. The temporal correlation between GLP-1 elevation and insulin secretion, combined with the GIFTLux control data, provides circumstantial evidence that effects are GLP-1-mediated, but more direct mechanistic evidence would strengthen this claim.

The HbA1c reduction over 30 days should be interpreted with appropriate caveats given that mouse erythrocyte lifespan is approximately 40-45 days. While substantial glycemic improvement over 30 days would be expected to produce measurable HbA1c changes weighted toward the treatment period, the timeframe is less than one complete erythrocyte turnover cycle. The concordance between fasting glucose reduction and HbA1c improvement supports biological plausibility, but this limitation warrants acknowledgment.

The use of oxygen-dependent reporters (GFP, luciferase) for visualizing bacterial activity in the largely anaerobic colonic environment is a technical limitation. While the Extended Data includes microaerobic growth conditions, these do not replicate colonic oxygen tension. The authors should clarify that these imaging data serve as visualization tools and that the primary evidence for in vivo function comes from oxygen-independent endpoints (serum GLP-1, blood glucose, insulin, HbA1c).

The statistical methods section specifies ANOVA with post-hoc tests but does not indicate the specific correction method applied for multiple comparisons. Given the large number of simultaneous statistical tests across the figures (particularly Figure 4 and Figure 6), explicit statement of the multiple comparison correction method is important for reproducibility and interpretation.

The antibiotic resistance markers (kanamycin, ampicillin) used for plasmid selection would require replacement for clinical translation, worthy of acknowledgement at minimum.

Version 3:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have addressed all my concerns

Referee #3

(Remarks to the Author)
First, I must admit that I am not the best reviewer for this paper. While we have been investigating a microbiome-based approach for diabetes therapy, this project goes far beyond any work I have done in animals. For instance, I am not qualified to address the statistical methods the authors employed. From my initial read, I thought the results Gao and colleague presented to be both fascinating and an encouraging novel new approach in diabetes treatment. The new version of the paper in view addresses the other reviewers' concerns effectively. The paper is ready to be viewed by the world. I think that some of the additional experiment proposed could be addressed in subsequent papers by this team.

Manuscript number: NATURE-2023-10-18581

Point-by-point response

We would like to thank the Editor and the Reviewers for their highly constructive comments. We have completed extensive additional experiments that collectively address all the points raised. 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 each of the reviewer comments (below). We start by listing the major experiments undertaken during our revision process.

1) We conducted additional *in vivo* experiments using wild-type mice to simulate the ON-OFF-ON and OFF-ON-OFF dynamics of gene expression by intraperitoneal injection of a glucose solution (1g/kg), demonstrating glucose-regulated gene expression dynamics of GIFT cells in mice (new **Fig. 2f-k** and **Supplementary Fig. 2, 3**).

2) We monitored GLP-1 levels in response to fluctuations in blood glucose in db/db mice. The results show that elevated blood glucose levels induce GLP-1 expression by GIFT cells and decrease as the blood glucose levels fall, demonstrating that GIFT cells can control protein expression in response to changes in glucose levels *in vivo* (new **Fig. 3d, e**).

3) We experimentally determined the effective concentration of GLP-1 released by the engineered bacteria and taken up by db/db mice, and compared it with that of a directly injected GLP-1 receptor agonist (semaglutide) (new **Supplementary Fig. 9c-e**). The results suggest that although the peak concentrations of GLP-1 achieved with our system are lower than those observed with direct injections of semaglutide, they are sufficient to provide a therapeutic effect through sustained, controlled expression.

4) We measured glucose concentrations in the gut lumen of wild-type and db/db mice, where GIFT cells temporarily reside following oral administration. Glucose levels were below 5 mM in wild-type mice and approximately 15 mM in db/db mice (new **Extended Data Fig. 5a**). These findings support that GIFT cells, when residing in the intestines of diabetic mice, are activated by elevated glucose levels.

5) We assessed the acute inflammatory and hypersensitivity reactions of $GIFT_{GLP\text{-}1}$ and semaglutide following a single dose administration, demonstrating significantly reduced acute adverse effects of $GIFT_{GLP\text{-}1}$ compared to semaglutide (new **Extended Data Fig. 10a-j** and **Supplementary Tables 1**, **2**).

6) We performed a conditioned taste aversion (CTA) study in db/db mice treated with $GIFT_{GLP\text{-}1}$ and semaglutide, demonstrating that semaglutide elicited a significantly greater aversive response compared with $GIFT_{GLP\text{-}1}$ (new **Extended Data Fig. 10k-n**).

7) We examined the risk of hypoglycemia when $GIFT_{GLP\text{-}1}$ and semaglutide were each combined with insulin glargine, and found that semaglutide combined with insulin glargine resulted in

significant increases in the incidence of hypoglycemia; no increased hypoglycemic events were observed when $GIFT_{GLP\text{-}1}$ was used in combination with insulin glargine (new **Supplementary Tables 3-9**).

8) We conducted a five-week study with spontaneous type 2 diabetic monkeys, demonstrating consistent glycemic control and a favorable safety profile of $GIFT_{GLP\text{-}1}$ throughout this observation window (new **Fig. 7g-l** and **Supplementary Tables 10-13**).

9) We conducted additional *in vivo* experiments using wild-type mice to assess the influence of oral administration of varying glucose concentrations on gene expression dynamics of $GIFT_{Lux}$ cells. Oral glucose induced gene expression, with expression levels increasing as the oral glucose concentration rose (new **Extended Data Fig. 9a-c** and **Supplementary Fig. 5**).

10) We assessed the effect of different dietary glucose content on sensor dynamics in wild-type mice, high-fat diet-induced obese (DIO) mice, and db/db mice. Consumption of high-sugar foods led to activation of gene expression in our GIFT cells, while low-sugar foods did not induce significant gene expression (new **Fig 5a-f, Extended Data Fig. 9d-f,** and **Supplementary Fig. 6-8**).

11) We monitored blood glucose levels of DIO and db/db mice receiving $GIFT_{GLP\text{-}1}$, after feeding mice with high-sugar items (*e.g.*, cola or chocolate). Oral $GIFT_{GLP\text{-}1}$ mitigated blood glucose fluctuations induced by intake of these items (new **Fig. 5g-k**).

12) We conducted a new mouse experiment to assess the therapeutic effect of $GIFT_{GLP\text{-}1}$ cells in db/db mice subjected to a low-carbohydrate diet. The experiment demonstrated that $GIFT_{GLP\text{-}1}$ cells remained effective in maintaining blood glucose homeostasis and contributing to body weight and fat mass reduction (new **Fig. 6a-h**).

13) We evaluated the therapeutic effect of $GIFT_{GLP\text{-}1}$ cells in high-fat diet-induced obese (DIO) mice and found that they did not exert glucose-lowering effects in mice with persistently normal blood glucose levels (new **Fig. 6i-p**).

14) We conducted new experiments in which DIO mice were exposed to streptozotocin to induce a nongenetic type 2 diabetes model. After diabetes model induction, mice were given $GIFT_{GLP\text{-}1}$ cells, $GIFT_{Lux}$ cells, or PBS: mice with $GIFT_{GLP\text{-}1}$ cells had significantly lower blood glucose levels, supporting the use of $GIFT_{GLP\text{-}1}$ cells to actively manage glucose levels in diabetic animals (new **Fig. 6q-x**).

15) We measured GIFT-regulated gene expression in response to various carbon sources, demonstrating its specificity as a glucose sensor (new **Extended Data Fig. 1d**).

16) We evaluated the performance of GIFT in simulated intestinal fluid (SIF)—including both aerobic and microaerobic contexts—illustrating GIFT's potential efficacy under conditions that mimic the gastrointestinal environment (new **Extended Data Fig. 1e**, **f**).

17) We encapsulated the bacteria with FDA-approved tannic acid (TA) and poloxamer 188 (F68), which extended the colonization duration of GIFT cells without adversely affecting bacterial growth or metabolism (new **Extended Data Fig. 3**).

18) We developed a construct for the expression of a GLP-1 tetra-copolymer in GIFT cells. Briefly, the homo-monomers of the GLP-1 tetra-copolymer are linked via enterokinase cleavage sites. This leads to an increased amount of active GLP-1 following cleavage by enterokinase (new **Supplementary Fig. 9a**, **b**).

19) We measured the protein levels and bioactivity of GLP-1 in cells after multiple rounds of sub-culturing, including constitutively expressed GLP-1 and GLP-1 secreted in response to glucose induction. There was a decline in both GLP-1 levels and bioactivity in the supernatant from constitutively expressing cells after multiple *in vitro* passages, whereas no such decrease was observed in glucose-induced GLP-1 expression cells. Additionally, plasmid sequencing from the 9th subculture of constitutive GLP-1 expression cells revealed the accumulation of genetic variants. These results suggest that inducible systems are more effective than constitutive systems for the secretion of GLP-1 (**Response Document Figure 3**).

Referee #1 (Remarks to the Author):

The authors engineer a probiotic to express GFP-1 in response to elevated glucose levels. They provide evidence for utilizing the system in mouse and NHPs with some therapeutic effects. The proof of principle seems to be demonstrated but unfortunately there are several deficiencies that severely restrict enthusiasm for this reviewer.

Reply:

We appreciate this reviewer's comments and constructive guidance.

Major concerns

* The novelty of the manuscript is unclear. The genetic circuitry to sense glucose is a glucose responsive promoter in *E. coli*, similar to those that have been used in the past, e.g. Courbet STM 2015, iGEM etc. There is not sufficient characterization in confounding environments.

Reply:

Thank you for your comments.

1. Novelty of the manuscript.

Novelty of the GIFT system: we would like to clarify the distinctions of our GIFT system compared to previously reported glucose sensors such as the pCpxP system and iGEM projects. Our GIFT system is based on the transcriptional repressor protein HexR from *Pseudomonas putida*, which senses glucose metabolites. In contrast, the glucose sensor developed by Courbet *et al.* in 2015 used the bacterial Cpx two-component system, and the iGEM sensors typically involve eukaryotic organisms (*e.g.*, mammalian cells), relying on endogenous signaling pathways.

The performance of the systems differs: The GIFT system demonstrates superior performance compared to the pCpxP system. Studies have shown that the pCpxP system exhibits relatively high basal expression levels in bacterial growth media and human serum, with a low signal-to-noise ratio (Courbet et al., *Sci Transl Med*, 2015). Moreover, the gene expression of the pCpxP system is inhibited by glucose concentrations greater than 10 mM. In contrast, our GIFT system is responsive at physiological concentrations of glucose (0-25 mM), exhibiting a substantial increase (48.8-fold over background) in sfGFP fluorescence when induced by 20 mM glucose. Additionally, the pCpxP system is activated by various carbon sources, indicating a lack of specificity (Lima et al., *Mol Microbiol*, 2011; **Response Document Figure 1a**). In contrast, our additional experiments demonstrate that GIFT responds minimally to other carbon sources found in physiological concentrations in the human gut or blood, with significant activation only by glucose (**Response Document Figure 1b**). This highlights the system's specificity and orthogonality to glucose.

The GIFT system and pCpxP system serve different purposes: GIFT, developed with the probiotic strain EcN, is designed as a live drug to monitor intestinal glucose levels in real-time and modulate glycemia by releasing protein drugs, enabling sense-and-response-based glycemic control upon oral administration. In contrast, the pCpxP system, utilizing the laboratory strain *E. coli* DH5α as a chassis, was designed for *ex vivo* detection in clinical samples (*e.g.*, serum, urine). To enhance the system's signal-to-noise ratio in detecting glucose, Courbet *et al.* (2015) introduced amplifying genetic

switches in their study, which increased sensitivity through BxB1 integrase. However, this approach is limited to recording transient signals and cannot achieve real-time quantitative detection of dynamic glucose changes. Thus, the GIFT system, as a probiotic glucose sensor developed for maintaining blood glucose homeostasis in diabetes, can be reasonably understood as a tool suitable for use in laboratory testing and for *in vivo* application as a live drug.

Additional new animal experiments in db/db mice, high-fat diet-induced obese (DIO) mice, nongenetic type 2 diabetic mice, and spontaneous diabetic monkeys validate the GIFT system's key advantages: (1) precise glucose-responsive gene expression with on-demand therapeutic release; (2) superior glycemic control via oral delivery; (3) improved metabolic regulation, including better insulin sensitivity, lipid profiles, and body weight management; and (4) fewer side effects, such as reduced hypoglycemia and lower inflammatory or hypersensitivity reactions compared to therapies like semaglutide. These advantages underscore the GIFT system's potential as an intelligent, live-drug platform for glycemic control.

Please see **page 5, lines 137-140** in the revised manuscript and below:

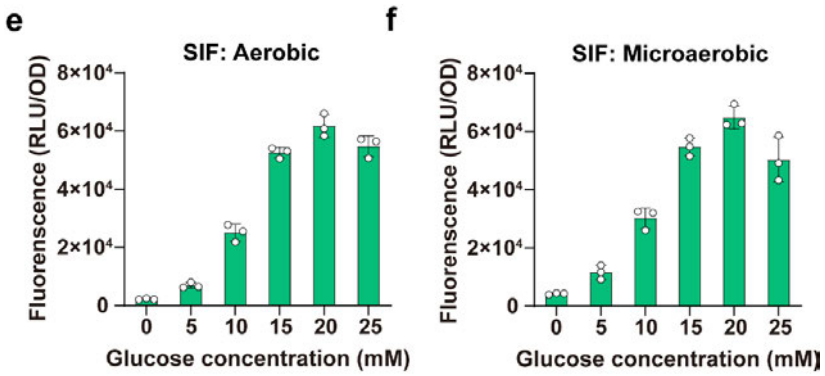
"We further assessed GIFT's response to various carbon sources and found significantly less activation by other carbon forms present at physiological concentrations in the human gut or blood compared to glucose (**Extended Data Fig. 1d**), highlighting its specificity as a glucose sensor."

[redacted]

2. We have also conducted additional experiments to address the concerns about system characterization in confounding environments.

Specifically, we have tested our system in simulated intestinal fluid (SIF), where we observed glucose-induced gene expression kinetics (new **Extended Data Fig. 1e**). Further, we have simulated the microaerobic environment of the intestine by sealing filled test tubes and allowing them to incubate statically. We found that our system can still be efficiently induced by glucose in microaerobic SIF (new **Extended Data Fig. 1f**), supporting its potential efficacy under conditions that mimic the gastrointestinal tract. Please see **page 5, lines 145-151** in the revised manuscript and below:

> "To the performance of GITF cells in a simulated human gastrointestinal environment, we tested $GIFT_{GFP}$ cells in simulated intestinal fluid (SIF). We observed glucose-induced gene expression kinetics (**Extended Data Fig. 1e**). Further, we mimicked the microaerobic conditions of the intestine by incubating sealed test tubes statically. Our results showed that the system remained effectively inducible by glucose in microaerobic SIF (**Extended Data Fig. 1f**), demonstrating its potential efficacy under conditions that mimic the gastrointestinal tract."



Response Document Figure 2 (see Extended Data Fig. 1e, f in the revised manuscript): Glucose-induced sfGFP expression from the glucose sensor in cells cultured in simulated human intestinal fluid (SIF) under aerobic (**e**) or microaerobic (**f**) conditions. Data are expressed as means ± SD; $n = 3$ independent experiments.

References:

Courbet A, Endy D, Renard E, Molina F, Bonnet J. Detection of pathological biomarkers in human clinical samples via amplifying genetic switches and logic gates. ***Sci Transl Med***. 2015.7(289):289ra83.

Lima BP, Antelmann H, Gronau K, Chi BK, Becher D, Brinsmade SR, Wolfe AJ. Involvement of protein acetylation in glucose-induced transcription of a stress-responsive promoter. ***Mol Microbiol***. 2011.81(5):1190-204.

* The authors cite the above reference 25 as "detect pathological biomarkers", but this is misleading given it is similarly glucose sensing. The authors claim their system as "self-feedback", which is also misleading as there is no true feedback control in the system, and the system also needs to be dosed every day.

Reply:

Thank you for your feedback. We recognize that a major contribution of the Courbet *et al.* study was the construction of a whole-cell biosensor designed to detect glucose concentrations in urine and serum, employing a glucose-sensing pCpxP system. Importantly, they enhanced the sensitivity and signal-to-noise ratio of this biosensor with an amplifying genetic switch. However, kindly note that while this system is adept at recording transient signals *ex vivo*, there was no demonstrated capacity for real-time quantitative monitoring of glucose dynamics, nor was any *in vivo* therapeutic potential shown. In response to your feedback, we have revised our manuscript to reflect the contributions of that study more accurately. Please see **page 3, lines 70-73** in the revised manuscript and below:

> "Engineered microbes have been used to detect pathological biomarkers and metabolites[24-26]. A notable example is the development of a whole-cell biosensor for detecting glucose concentrations in urine and serum, utilizing a glucose-sensing pCpxP system[25]."

We now understand the problems with our previous "self-feedback" diction (*i.e.*, not an informative description of the operational dynamics of the GIFT system as currently configured). We have revised our manuscript to better describe the system as having a "functional response" rather than "self-feedback." Please note that we have now named our system "glucose sensing and functional response probiotic (GIFT)."

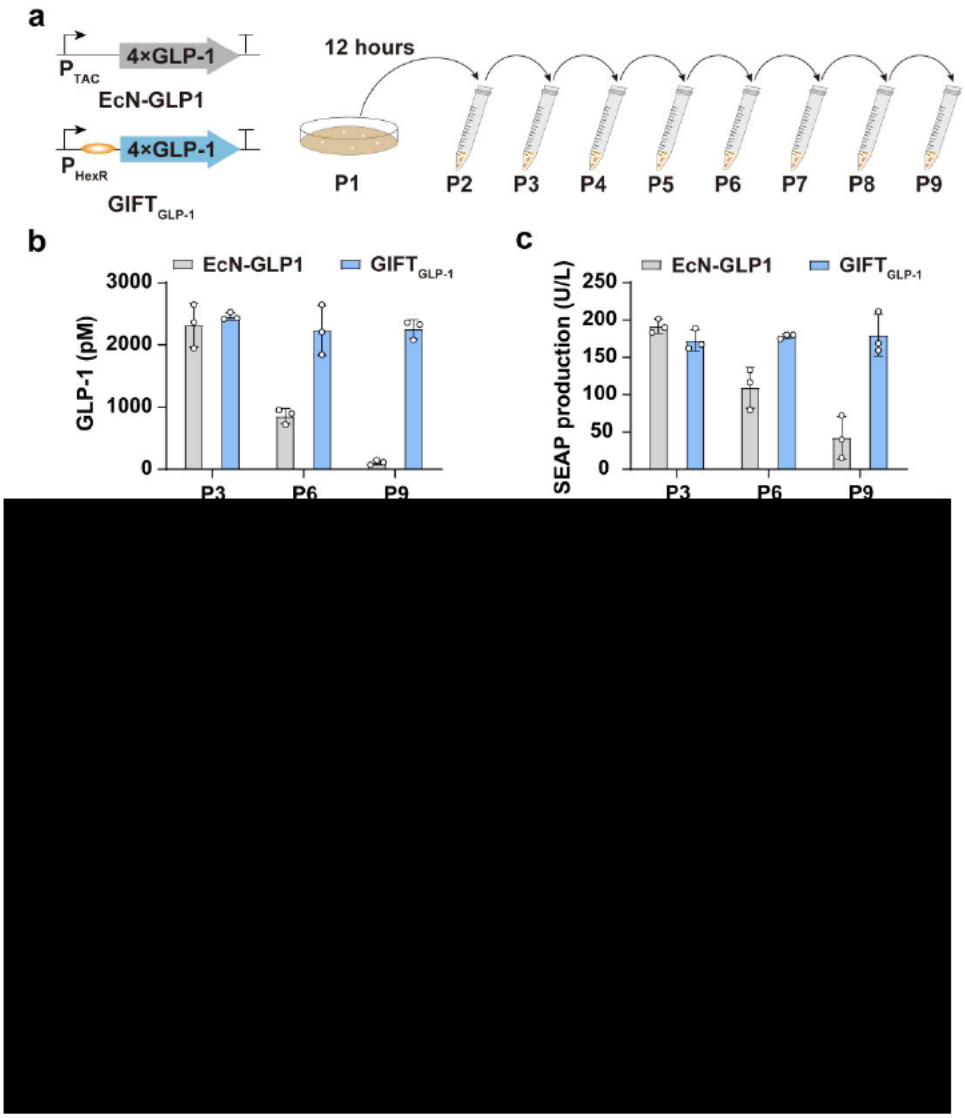
* A discussion of other efforts on engineered bacteria for treatment of diabetes is critically missing. For example, work performed in 2014 by Agarwal (DOI 10.1007/s11095-014-1430-3) using *L. lactis* similarly showed a constitutively expressed GLP-1 was also therapeutic, in the absence of sensing-mediated control. In Guan et al., the authors do not demonstrate the advantage over a constitutively expressed GLP, limiting the strength of claiming that such an engineered platform was required or beneficial beyond a simpler system, or its safety benefits.

Reply:

Thank you for your comments.

1. We appreciate the reference to Agarwal's study as it highlights an alternative approach where therapeutic efficacy was achieved without the complexity of a regulated system. We conducted new experiments to assess the differences between glucose-induced ($GIFT_{GLP-1}$) and constitutive GLP-1 expression in EcN cells (EcN-GLP1). ELISA results demonstrated a progressive reduction in GLP-1 levels in the supernatant of EcN-GLP1 after multiple *in vitro* passages (**Response Document Figure 3a**), with a near-complete absence of the protein after several generations (**Response Document Figure 3b**). Moreover, activity assays indicated a decline in the functionality of the secreted GLP-1, suggesting a loss of biological activity over time (**Response Document Figure 3c**). Sequencing analysis of the plasmids from the 9th subculture of this strain also showed large deletions or frameshift mutations within the protein-encoding genes (**Response Document Figure 3d**). In contrast, and using the same conditions, the gene expression of $GIFT_{GLP-1}$ remained stable throughout the observation window (**Response Document Figure 3b**,**c**). We interpret these findings as aligning with a (to our understanding) broader consensus that inducible systems are more effective than constitutive systems for the secretion of exogenous functional proteins, as they can better manage protein expression levels

and reduce the metabolic burden on host cells (Snoeck et al., *Microb Cell Fact*, 2024; Browning et al., *Nat Rev Microbiol*, 2004).



Response Document Figure 3: A comparison between inducible and constitutive expression of GLP-1. a, Schematic of the time schedule for sub-culturing the engineered strains. GIFT$_{GLP-1}$ cells and EcN transformed with a plasmid for constitutive GLP-1 expression (EcN-GLP1) were cultured in LB medium at 37 °C, with a 1% inoculum sub-cultured every 12 hours. **b**, ELISA for GLP-1 secreted by EcN-GLP1 and GIFT$_{GLP-1}$ cells across different generations. EcN-GLP1 cells and GIFT$_{GLP-1}$ cells from the 3rd, 6th, and 9th subcultures were inducted with 15 mM glucose for 8 hours, and the culture supernatant was collected for ELISA analysis. **c**, Bioactivity of the secreted GLP-1 from EcN-GLP1 and GIFT$_{GLP-1}$ cells across different generations. EcN-GLP1 cells and GIFT$_{GLP-1}$ cells from the 3rd, 6th, and 9th subcultures were inducted with 15 mM glucose for 8 hours, and the culture supernatant was supplemented to HEK293T cells expressing the GLP-1 receptor (GLP1R) and a SEAP reporter driven by a P$_{CRE}$ promoter.

2. Sustained high dosages of GLP-1 have been shown to lead to adverse effects, a phenomenon repeatedly verified in clinical settings (Sodhi et al., *JAMA*, 2023). Our developed sensor system is designed to dynamically regulate GLP-1 expression in the gastrointestinal tract, thus minimizing

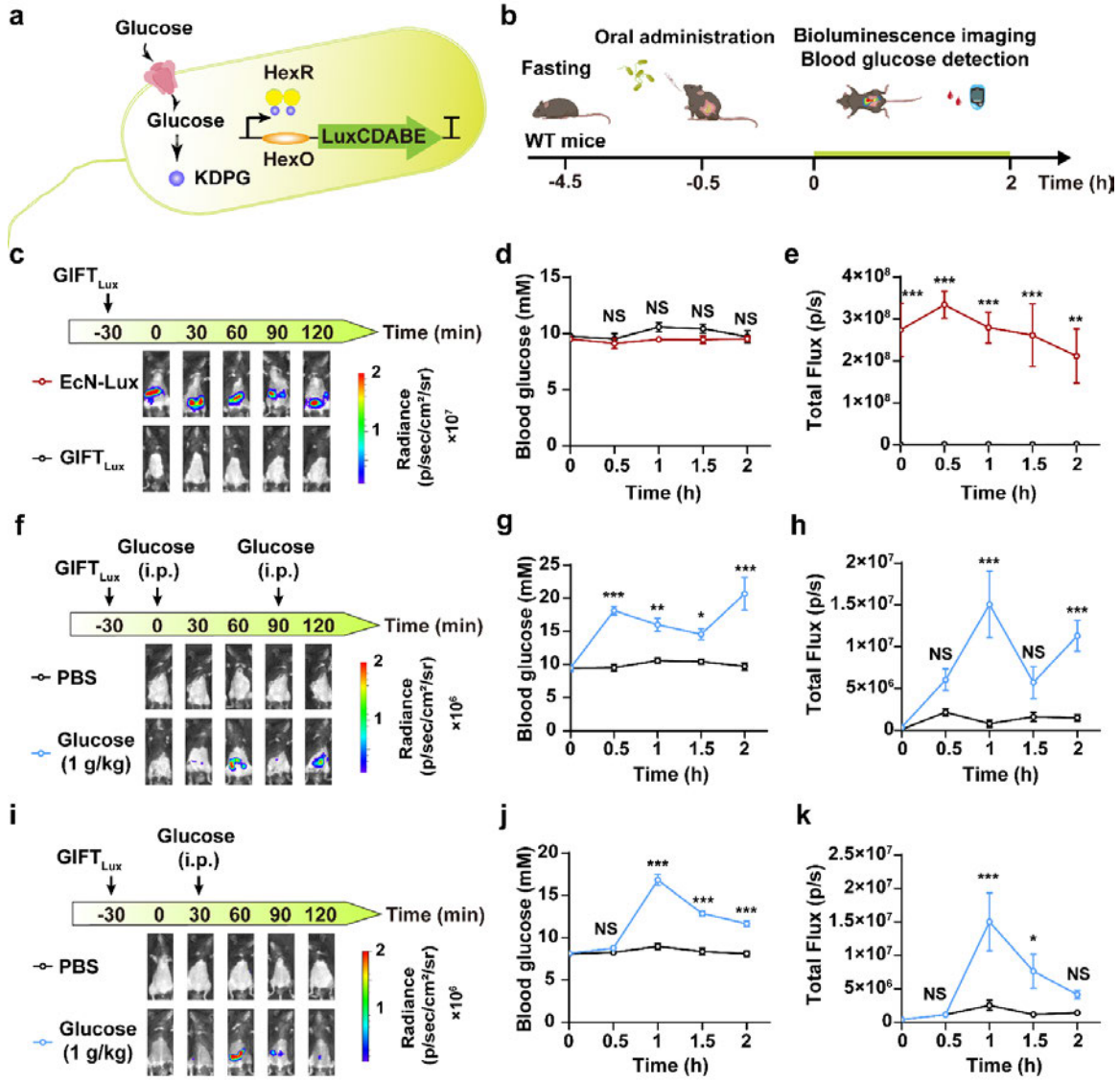
potential side effects. To compare the gene expression dynamics by $GIFT_{Lux}$ and EcN cells that constitutively expressed LuxCDABE (EcN-Lux), we conducted additional *in vivo* experiments using wild-type mice. Without glucose induction, the gene expression of GIFT cells in wild-type mice was significantly lower than that of EcN-Lux (new **Fig. 2c-e** and **Supplementary Fig. 1**).

We then administered the bacteria to the mice via oral gavage and simulated the ON-OFF-ON dynamics of gene expression by intraperitoneal injection of a glucose solution (1 g/kg) (new **Fig. 2f-h** and **Supplementary Fig. 2**). Specifically, 30 minutes post-gavage, we injected the glucose solution intraperitoneally to elevate the blood glucose levels in the mice, which resulted in the activation of LuxCDABE expression. As the blood glucose levels decreased, the expression of LuxCDABE also diminished. Further glucose injections reactivated the expression of LuxCDABE, demonstrating the system's ability to respond dynamically to changes in glucose concentration. Similar OFF-ON-OFF dynamics were observed in mice (new **Fig. 2i-k** and **Supplementary Fig. 3**). These results illustrate that our GITF cells can control protein expression in response to changes in glucose levels.

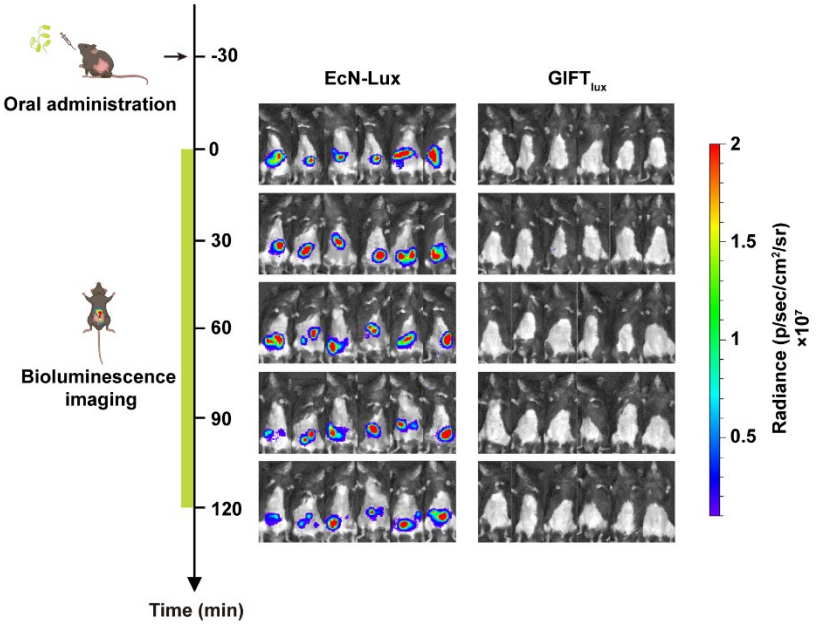
These new findings have been added to our revised manuscript. Please see **page 7, lines 188-204** in the revised manuscript and below:

"*Photorhabdus luminescens* LuxCDABE was used as the output of GIFT, and the engineered $GIFT_{Lux}$ cells (**Fig. 2a**) were orally delivered into wild-type mice (10^9 CFU/mouse) to assess *in vivo* LuxCDABE expression (**Fig. 2b**). Compared to mice treated with EcN cells constitutively expressing LuxCDABE (EcN-Lux), $GIFT_{Lux}$-treated normoglycemic wild-type mice showed significantly lower bioluminescence signals (**Fig. 2c-e** and **Supplementary Fig. 1**), indicating that the GIFT cannot be activated in normoglycemic conditions.

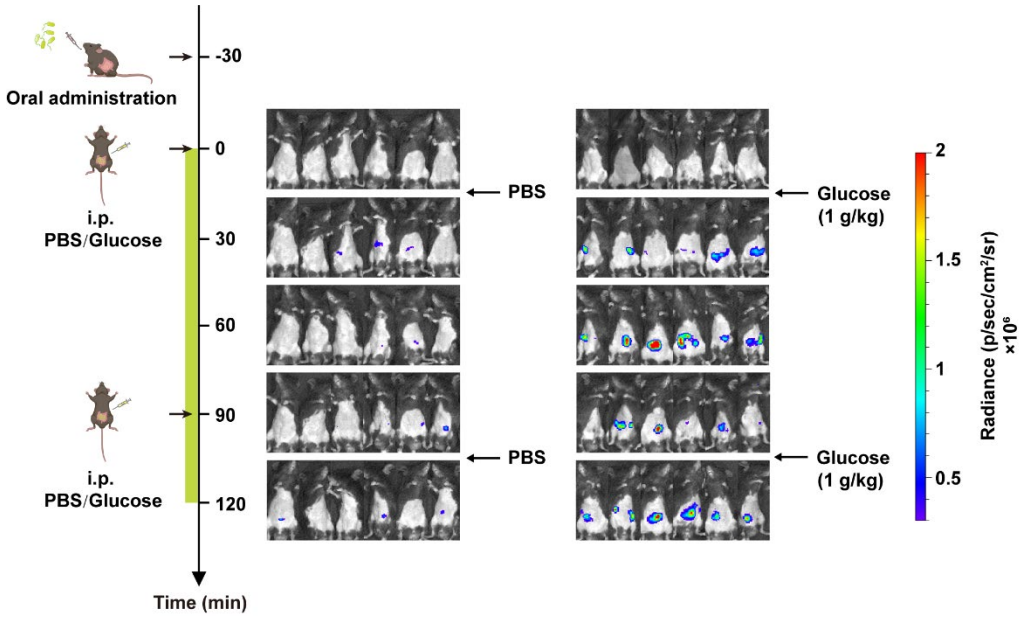
To explore the ON-OFF-ON gene expression dynamics, we simulated blood glucose fluctuations typically seen in diabetic mice through intraperitoneal glucose injections. Thirty minutes after oral administration of $GIFT_{Lux}$ cells, glucose solution (1 g/kg) was injected intraperitoneally to elevate blood glucose, activating LuxCDABE expression in $GIFT_{Lux}$-treated mice. As blood glucose levels dropped, LuxCDABE expression decreased accordingly. The second glucose injection reactivated LuxCDABE expression, demonstrating the system's ability to dynamically respond to glucose fluctuations (**Fig. 2f-h** and **Supplementary Fig. 2**). Similar OFF-ON-OFF gene expression dynamics were observed in mice orally delivered with $GIFT_{Lux}$ (**Fig. 2i-k** and **Supplementary Fig. 3**). These findings demonstrate that GIFT cells can modulate protein expression in response to changing glucose levels."



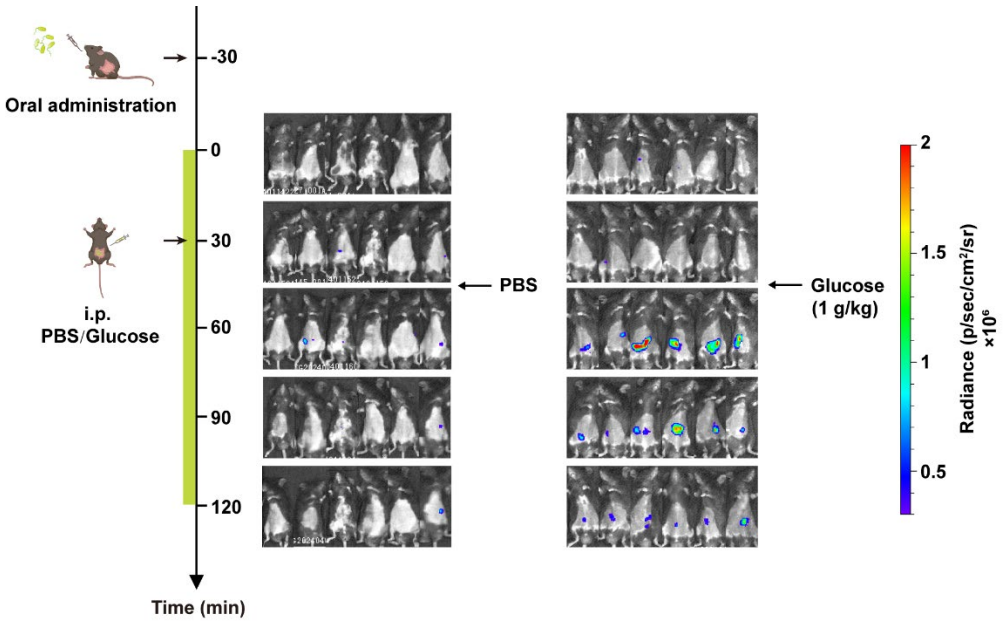
Response Document Figure 4 (see Fig. 2 in the revised manuscript): GIFT-mediated gene expression kinetics after oral delivery to mice. a, Schematic of glucose-triggered LuxCDABE expression in GIFT cells. **b**, Timeline for GIFT-mediated gene expression in wild-type mice. After a 4-hour fast, mice were orally administered GIFT cells expressing LuxCDABE ($GIFT_{Lux}$) and subjected to various treatments. Blood glucose and bioluminescence signals were profiled over 2 hours. **c-e**, Kinetics of constitutive and GIFT-mediated gene expression in wild-type mice. Mice received oral doses of EcN constitutively expressing LuxCDABE (EcN-Lux) or $GIFT_{Lux}$ cells (10^9 CFU). Blood glucose (**d**) and bioluminescence signals (**c**, **e**) were measured every 30 min using an *in vivo* imaging system. **f-h**, GIFT-mediated gene expression kinetics (ON-OFF-ON) in wild-type mice. Mice were orally administered $GIFT_{Lux}$ cells (10^9 CFU) at -30 min, and intraperitoneally injected (i.p.) with PBS or glucose (1 g/kg) at 0 and 90 min. Blood glucose (**g**) and bioluminescence signals (**f**, **h**) were recorded every 30 min. **i-k**, GIFT-mediated gene expression kinetics (OFF-ON-OFF) in wild-type mice. Mice received $GIFT_{Lux}$ cells (10^9 CFU) at -30 min, and intraperitoneally injected (i.p.) with PBS or glucose (1 g/kg) at 30 min. Blood glucose (**j**) and bioluminescence signals (**i**, **k**) were recorded every 30 min. Data in **d**, **e**, **g**, **h**, **j**, and **k** are expressed as means ± SEM; $n = 6$ mice. *P*-values were calculated by two-way ANOVA. NS, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.



Response Document Figure 5 (see Supplementary Fig. 1 in the revised manuscript): Constitutive and GIFT-mediated gene expression kinetics in wild-type mice. After a 4-hour fast, wild-type mice were orally administered either EcN constitutively expressing LuxCDABE (EcN-Lux) or GIFT$_{Lux}$ cells (10^9 CFU). Imaging was performed every 30 min using an *in vivo* imaging system.



Response Document Figure 6 (see Supplementary Fig. 2 in the revised manuscript): GIFT-mediated gene expression kinetics (ON-OFF-ON) in wild-type mice. After a 4-hour fast, wild-type mice were orally administered GIFT$_{Lux}$ cells (10^9 CFU) at -30 min, followed by intraperitoneal injection (i.p.) of PBS or glucose (1 g/kg) at 0 and 90 min. Imaging was performed every 30 min using an *in vivo* imaging system.



Response Document Figure 7 (see Supplementary Fig. 3 in the revised manuscript): GIFT-mediated gene expression kinetics (OFF-ON-OFF) in wild-type mice. After a 4-hour fast, wild-type mice were orally administered $GIFT_{Lux}$ cells (10^9 CFU) at -30 min, followed by intraperitoneal injection (i.p.) of PBS or glucose (1 g/kg) at 30 min. Imaging was performed every 30 min using an *in vivo* imaging system.

In response to the concerns raised, we have expanded the discussion in our manuscript to include the ongoing efforts using engineered bacteria for the treatment of diabetes, and provide a content regarding the preference for inducible systems over constitutive systems for the design of many engineered bacteria aimed at therapeutic applications. Please see **page 19, lines 541-545** in the revised manuscript and below:

> "Several efforts have been made to develop oral-engineered bacteria for GLP-1 expression, showing therapeutic potential in diabetes and obesity in mouse models[70,71]. However, constitutive expression often leads to low protein levels, imposes a metabolic burden on host bacteria[72], and lacks the ability for real-time on-demand release, compromising protein stability and clinical effectiveness."

References:

Sodhi M, Rezaeianzadeh R, Kezouh A, Etminan M. Risk of gastrointestinal adverse events associated with glucagon-like peptide-1 receptor agonists for weight loss. JAMA. 2023. 330(18): 1795-1797.

Browning DF, Busby SJ. The regulation of bacterial transcription initiation. Nat Rev Microbiol. 2004. 2(1): 57-65.

Snoeck S, Guidi C, De Mey M. "Metabolic burden" explained: stress symptoms and its related responses induced by (over)expression of (heterologous) proteins in *Escherichia coli*. Microb Cell Fact. 2024. 23(1): 96.

* Given the inability of EcN to colonize long-term, and need for daily dosing at high levels, utility of the system is unclear. Motivation lists long-term dosing with high levels of GLP can cause adverse

effects. So then, as a probiotic releasing GLP recombinantly, could also generate similar effects. It is critical to compare the author's effective concentration released by the bacteria and taken up by the subjects to establish a translational advantage to constitutive or other SOCs. What would be the concentration of direct GLP-1 delivery be, and how do dose-response effects in this or probiotic mediated systems differ.

Reply:

Thank you for your insightful comments.

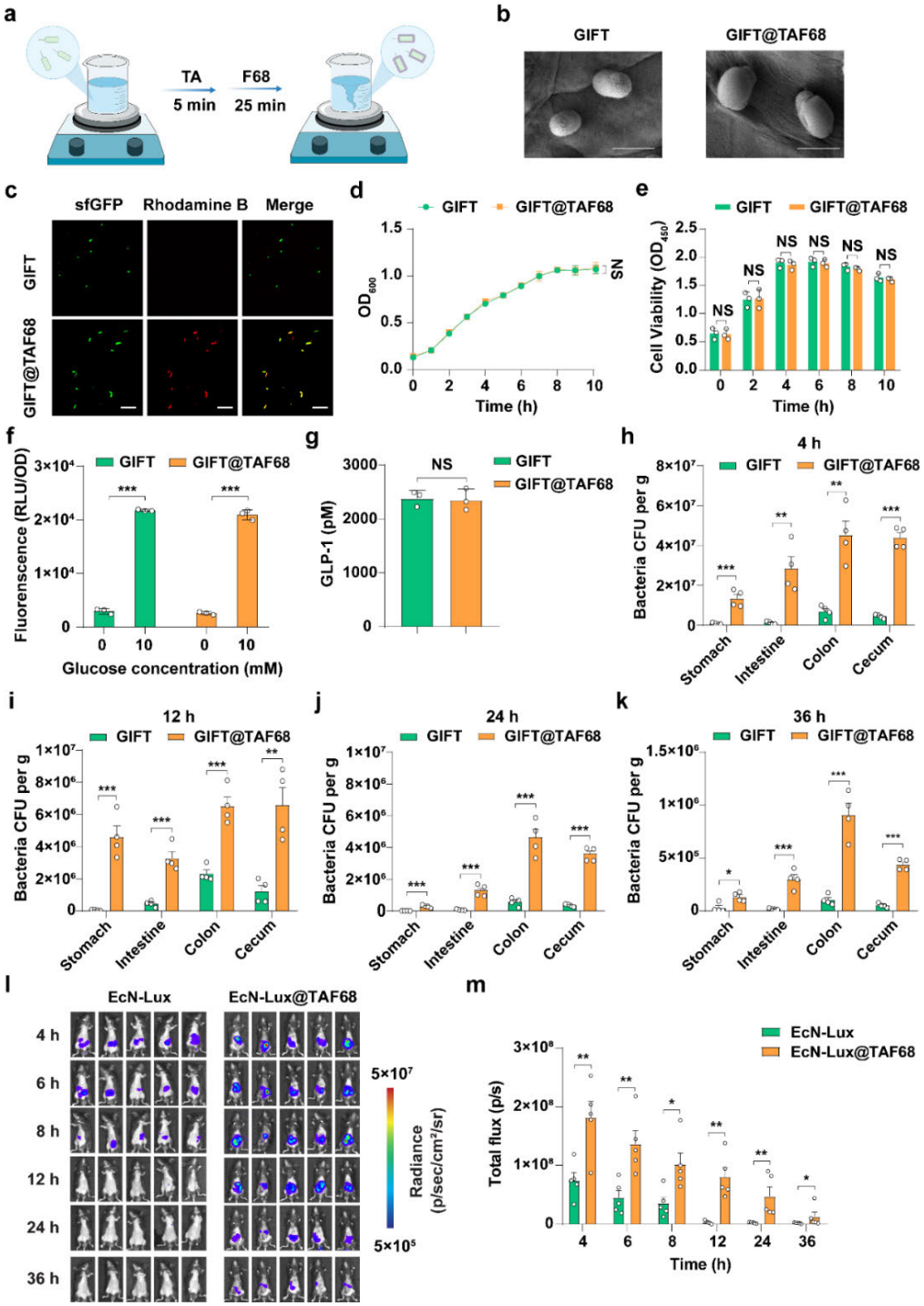
1. We recognize that the inherent inability of EcN to colonize the gut long-term requires frequent administration. However, kindly note that the dosing frequency for our oral bacterial system is consistent with clinically safe doses that have been validated in multiple clinical studies (Isabella et al., *Nat Biotechnol*, 2018; Russell et al., *Cell*, 2022; Perreault et al., *Cell Host Microbe*, 2024). Additionally, reports in the literature frequently assert that an oral route of administration is more user-friendly than injections, which are the typical method for most GLP-1 receptor agonists currently in use. Indeed, a major reason for the high discontinuation rates of GLP-1 receptor agonist therapies is the difficulty patients face with regular injections, underscoring the global effort towards developing oral GLP-1 therapies (Holman et al., *N Engl J Med*, 2017; Drucker, *Nat Rev Drug Discov*. 2020; Saxena et al., *Nat Med*, 2021). Moreover, we believe that engineered bacterial therapies, unlike long-term microbiota transplants, do not require ongoing customization. Further, we argue that the inability of EcN to colonize long-term should not be viewed solely as a drawback; instead, it can enhance safety and controllability (Kurtz et al., *Sci Transl Med*, 2019). Specifically, the transient nature of EcN colonization allows for temporal control over therapeutic interventions. Should any adverse effects arise, the effects of the cells can rapidly cease, thus allowing for immediate management of potential risks. Additionally, we consulted with clinical practitioners, who affirmed that a daily oral administration format is patient-friendly, easy to remember, and less prone to confusion in clinical practice.

We would also like to highlight that we have now completed new experiments exploring methods to extend colonization duration (which could, for example, minimize the dosage and frequency of administration). Specifically, we encapsulated the bacteria with tannic acid (TA) and poloxamer 188 (F68), which are FDA-approved pharmaceutical excipients (**Extended Data Fig. 3a-c**). This encapsulation significantly enhanced the intestinal colonization duration of the bacteria from 8 to 36 hours (**Extended Data Fig. 3h-m**), without adversely affecting bacterial growth or metabolism (**Extended Data Fig. 3d-g**). To further improve drug delivery efficiency, we developed a construct for expressing a tetra-copolymer of GLP-1 in GIFT cells. Briefly, the homo-monomers of the GLP-1 tetra-copolymer are linked via enterokinase cleavage sites. This design ensures that upon exposure to enterokinase, which is naturally present in the gut, the tetra-copolymer is efficiently cleaved into active GLP-1 monomers (new **Supplementary Fig. 9a**). *In vitro* experiments demonstrate that this configuration significantly enhances the production of active GLP-1 following cleavage by enterokinase (new **Supplementary Fig. 9b**). Please see **page 6, lines 170-176** and **page 13, lines 379-383** in the revised manuscript and below:

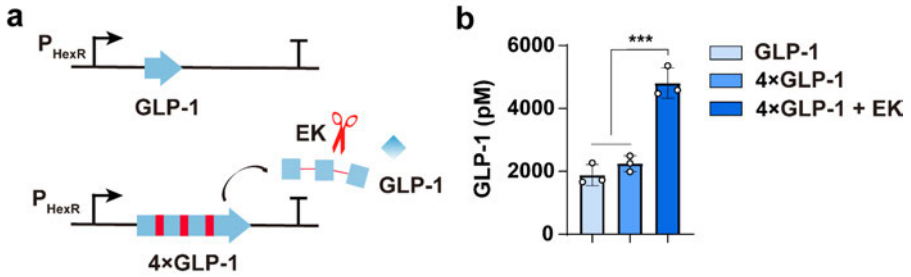
> "To prolong the residence time of GIFT cells in the gastrointestinal tract, we encapsulated the cells with tannic acid (TA) and poloxamer 188 (F68), both FDA-approved excipients[43] (**Extended Data Fig. 3a-c**). This encapsulation did not negatively impact bacterial growth or

metabolism (**Extended Data Fig. 3d, e**), nor did it affect system dynamics or therapeutic protein secretion (**Extended Data Fig. 3f, g**). Additionally, encapsulation with TA and F68 extended the intestinal colonization of GIFT cells in mice from 8 to 36 hours, as shown by *in vivo* imaging (**Extended Data Fig. 3h-m**)."

"To improve drug delivery, we engineered GIFT$_{GLP-1}$ cells to express a GLP-1 tetra-copolymer, with its monomers linked by enterokinase cleavage sites, allowing activation by enterokinase, an enzyme naturally present in the gut (**Supplementary Fig. 9a**). *In vitro* tests showed that this design significantly increased active GLP-1 production following cleavage by enterokinase (**Supplementary Fig. 9b**)."



Response Document Figure 8 (see Extended Data Fig. 3 in the revised manuscript): Characterization of GIFT intestinal colonization coated with tannic acid (TA) and poloxamer 188 (F68). **a**, Schematic representation of the preparation process for coated GIFT (GIFT@TAF68). $GIFT_{GFP}$ cells were sequentially treated with TA and F68 for 5 min, followed by an additional 25 min of F68 treatment with continuous stirring throughout the encapsulation process. **b**, Scanning electron microscope (SEM) images of uncoated $GIFT_{GFP}$ and $GIFT_{GFP}$@TAF68. Scale bar, 1 μm. **c**, Confocal scanning laser microscopy (CSLM) images of $GIFT_{GFP}$ and $GIFT_{GFP}$@TAF68. The red channel represents rhodamine B-labeled TA, while the green channel shows sfGFP expressed by $GIFT_{GFP}$. Scale bar, 10 μm. **d**, Growth curves of $GIFT_{GFP}$ and $GIFT_{GFP}$@TAF68 cultured in LB broth at 37 °C. OD_{600} was recorded using a microplate reader. **e**, Cell viability of $GIFT_{GFP}$ and $GIFT_{GFP}$@TAF68. **f**, Quantification of sfGFP expression by $GIFT_{GFP}$ and $GIFT_{GFP}$@TAF68 after 12 hours of culture with 10 mM glucose. **g**, Measurement of GLP-1 secretion by $GIFT_{GLP\text{-}1}$ and $GIFT_{GLP\text{-}1}$@TAF68 after 12 hours of culture with 15 mM glucose. **h-k**, Bacterial colony counts in the gastrointestinal tract of mice. C57BL/6 mice were orally administrated 10^9 CFU of either $GIFT_{GFP}$ or $GIFT_{GFP}$@TAF68 cells. Tissue samples from the stomach, small intestine, colon, and cecum were harvested at 4 (**h**), 12 (**i**), 24 (**j**), and 36 hours (**k**) post-administration, samples were homogenized and plated on LB agar. After 12 hours of incubation at 37 °C, bacterial colonies were counted. **l**, Bioluminescence images of mice. EcN constitutively expressing LuxCDABE (EcN-Lux) (10^9 CFU) were orally administrated to C57BL/6 mice, and imaging was performed at 4, 6, 8, 12, 24, and 36 hours using an *in vivo* imaging system. **m**, Quantification of bioluminescence signals from **l**. Data in **d-g** are expressed as means ± SD; $n = 3$ independent experiments. Data in **h-k** and **m** are expressed as means ± SEM; $n = 4$ mice for **h-k**, $n = 5$ mice for **m**. *P*-values were calculated using a two-tailed unpaired *t*-test. NS, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

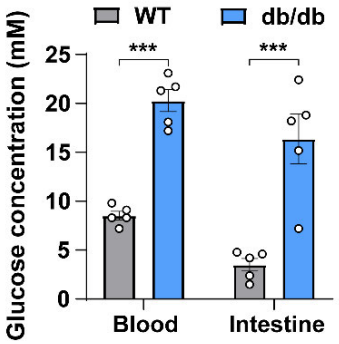


Response Document Figure 9 (see Supplementary Fig. 9 in the revised manuscript): Design of a construct for GLP-1 tetra-copolymer expression in GIFT cells. **a**, Schematic of the genetic configurations for GLP-1 and tetrameric GLP-1 (4×GLP-1) expression systems. The 4×GLP-1 is linked via enterokinase (EK) cleavage sites. **b**, Quantification of GLP-1 levels in the culture supernatant from $GIFT_{GLP\text{-}1}$ cells expressing either GLP-1 or 4×GLP-1. Cells (10^9 CFU) were induced with 15 mM glucose for 12 hours, followed by incubation of the supernatant with enterokinase for 12 hours to activate tetrameric GLP-1. GLP-1 content was then measured using an ELISA kit. Data in **b** are expressed as means ± SD; $n = 3$ independent experiments. *P*-values were calculated using a two-tailed unpaired *t*-test. $***P < 0.001$.

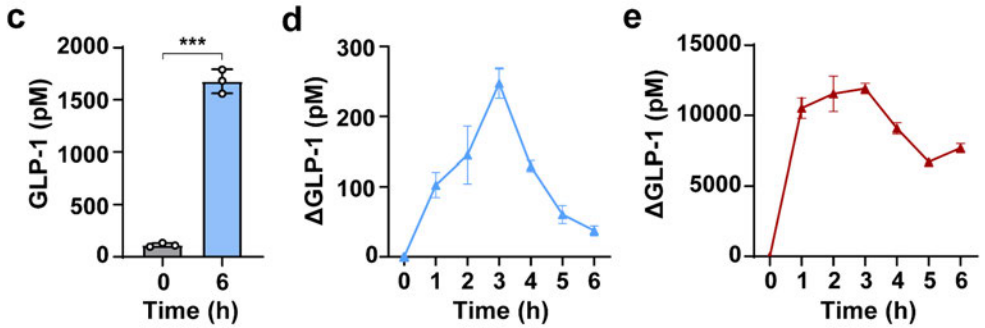
2. In response to your guidance, we conducted additional studies to compare the effective concentration of GLP-1 released by our bacteria and taken up by db/db mice with that of a directly injected GLP-1 receptor agonist, semaglutide. Initially, we measured intestinal glucose concentrations in 8-week-old db/db mice, which are approximately 15 mM (new **Extended Data Fig. 5a**). Then, we measured the

amount of GLP-1 released by $GIFT_{GLP-1}$ cells by simulating the glucose-induced scenario within the gastrointestinal tract. Specifically, $GIFT_{GLP-1}$ cells (10^9 CFU) were cultured in simulated intestinal fluid supplied with 15 mM glucose under microaerobic conditions for 6 hours, and the GLP-1 secreted in the culture supernatant was measured using an ELISA kit (new **Supplementary Fig. 9c**). We also monitored the increased serum GLP-1 levels of db/db mice following administration of $GIFT_{GLP-1}$ every hour for 6 hours, which ranged from 40 to 250 pM (new **Supplementary Fig. 9d**). Based on these data, we calculated the bioavailability of GLP-1 mediated by GIFT to be approximately 10.5%. Moreover, we measured the active GLP-1 in db/db after semaglutide injection (0.12 mg/kg), which ranged from 6,000 to 12,000 pM within 6 hours (new **Supplementary Fig. 9e**). These findings suggest that although the serum levels of GLP-1 achieved with GIFT cells are significantly lower than those with direct injections of semaglutide, they are nonetheless sufficient to provide a therapeutic effect. Please see **page 15, lines 430-445** in the revised manuscript and below:

> "To evaluate the translational potential of $GIFT_{GLP-1}$ cells, we compared them to semaglutide[62,63], a clinically approved GLP-1 receptor agonist for type 2 diabetes and obesity, typically administered as a once-weekly injection. Our initial focus was on the bioavailability of GLP-1 mediated by GIFT compared to directly injected semaglutide. To quantify the GLP-1 release from $GIFT_{GLP-1}$ cells, we simulated a glucose-induced environment in the gastrointestinal tract. $GIFT_{GLP-1}$ cells (10^9 CFU) were cultured in simulated intestinal fluid with 15 mM glucose (**Extended Data Fig. 5a)** under microaerobic conditions for 6 hours. GLP-1 levels in the culture supernatant were then measured using an ELISA kit (**Supplementary Fig. 9c**). Based on the serum GLP-1 levels observed in db/db mice, which ranged from 40 to 250 pM over 6 hours (**Supplementary Fig. 9d**), we estimated the bioavailability of GLP-1 mediated by GIFT to be approximately 10.5%. In comparison, active GLP-1 levels in db/db mice treated with semaglutide (0.12 mg/kg) ranged from 6,000 to 12,000 pM over 6 hours (**Supplementary Fig. 9e**). These findings indicate that while serum GLP-1 levels achieved Although the numbers of GIFT cells are significantly lower than those from direct semaglutide injections, they are still sufficient to exert a therapeutic effect".



Response Document Figure 10 (see Extended Data Fig. 5a in the revised manuscript): Measurement of intestinal glucose concentrations in wild-type and db/db mice. Following a 4-hour fasting period, the blood glucose levels of the mice were measured. The mice were then euthanized, and their intestines were harvested; the mucosal lining of the intestines was collected. Glucose content was measured using a commercial glucose assay kit. Data are expressed as means ± SEM; $n = 5$ mice. *P*-values were calculated by two-way ANOVA. ***$P < 0.001$.



Response Document Figure 11 (see Supplementary Fig. 9c-e in the revised manuscript): Measurement of serum GLP-1 levels in db/db mice. c, Measurement of GLP-1 released by $GIFT_{GLP-1}$ cells in a simulated glucose-induced gastrointestinal environment. $GIFT_{GLP-1}$ cells (10^9 CFU) were cultured in simulated intestinal fluid with 15 mM glucose under microaerobic conditions for 6 hours, and the secreted GLP-1 in the supernatant was quantified using an ELISA kit. **d**, **e**, Increased serum GLP-1 levels in db/db mice following oral administration of $GIFT_{GLP-1}$ cells (**d**) or semaglutide injection (**e**). $GIFT_{GLP-1}$ cells (10^9 CFU) or semaglutide (0.12 mg/kg) were administered to db/db mice, and serum GLP-1 levels were monitored for 6 hours. The increase in GLP-1 relative to baseline (0 hours) was calculated. Data in **c** are expressed as means ± SD; $n = 3$ independent experiments. Data in **d** and **e** are expressed as means ± SEM; $n = 5$ mice. *P*-values were calculated using a two-tailed unpaired *t*-test (**c**). $***P < 0.001$.

3. Numerous studies and clinical trials have highlighted a range of adverse effects associated with the systemic administration of GLP-1 receptor agonists, such as gastrointestinal discomfort, pancreatitis, pancreatic cancer, and thyroid complications, among others (Sodhi et al., *JAMA*, 2023; Gier et al., *Diabetes*, 2012; Gier, *Gastroenterology*. 2011; Gier et al., *J Clin Endocrinol Metab*, 2012). To extend the half-life of GLP-1 receptor agonists, these agents are often modified with lipid-like structures. While these modifications successfully improve drug efficacy, they may also lead to the development of resistance or allergic reactions (Liu et al., *J Pharmacol Toxicol Methods*, 2011; Garay et al., *Expert Opin Drug Deliv*, 2012; Schellekens, *Pharm Res*. 2013). Our engineered probiotic system is specifically designed to mitigate these risks by providing controlled expression of GLP-1 on demand, thereby potentially reducing overall exposure compared to systemic delivery. To evaluate the potential adverse effects associated with systemic semaglutide (a clinically approved GLP-1 receptor agonist used for the treatment of type 2 diabetes and obesity, administered as a once-weekly injectable) injections versus the targeted, controlled delivery of GLP-1 via GIFT, we conducted a series of evaluations in db/db mice.

First, to assess acute inflammatory and hypersensitivity reactions, blood samples of db/db mice receiving a single administration of semaglutide or $GIFT_{GLP-1}$ were collected for complete blood count, serum biochemical analysis, and analysis of immunoglobulin E (IgE) and calcitonin levels (new **Extended Data Fig. 10a** and **Supplementary Tables 1**,**2**). Semaglutide (1 mg/kg) injection led to a host of physiological impacts, none of which were observed for the mice given oral GIFT administration. Specifically, the semaglutide group mice had significantly elevated specific IgE levels at 6 hours after dosing, indicative of an immediate hypersensitivity reaction (new **Extended Data Fig. 10b**). Increases in plasma calcitonin levels were observed at 12 and 24 hours after a single dose of

semaglutide, suggesting potential adverse effects on the thyroid gland (new **Extended Data Fig. 10c**, **d**). Additionally, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly elevated at 12 hours after semaglutide injection but returned to normal levels by 24 hours, indicating an acute hepatic stress response (new **Extended Data Fig. 10e-h**). Moreover, serum IL-6 and IFN-β levels were significantly increased at 6 hours after injection, indicating an acute inflammatory response (**Extended Data Fig. 10i**, **j**).

We also performed a conditioned taste aversion (CTA) study in db/db mice to investigate the adverse responses elicited by $GIFT_{GLP\text{-}1}$ and semaglutide (new **Extended Data Fig. 10k**). CTA is a highly specialized form of associative learning in which animals learn to avoid a taste that is associated with a detrimental health condition, demonstrating its utility for assessing potential aversive effects of treatments in a medical context (Garcia et al., *Science*, 1955). The mice were exposed to a highly palatable 1% sucrose solution, followed by administration of $GIFT_{GLP\text{-}1}$ (10^9 CFU) or semaglutide (0.12 mg/kg or 1 mg/kg). After the administration, the mice were then exposed to a two-bottle taste preference test, offering a voluntary choice between tap water and a 1% sucrose solution. The consumption of sucrose and water was monitored for 24 hours. Compared to $GIFT_{GLP\text{-}1}$, we found that semaglutide induced a significantly greater aversive response to the sucrose solution (new **Extended Data Fig. 10l-n**).

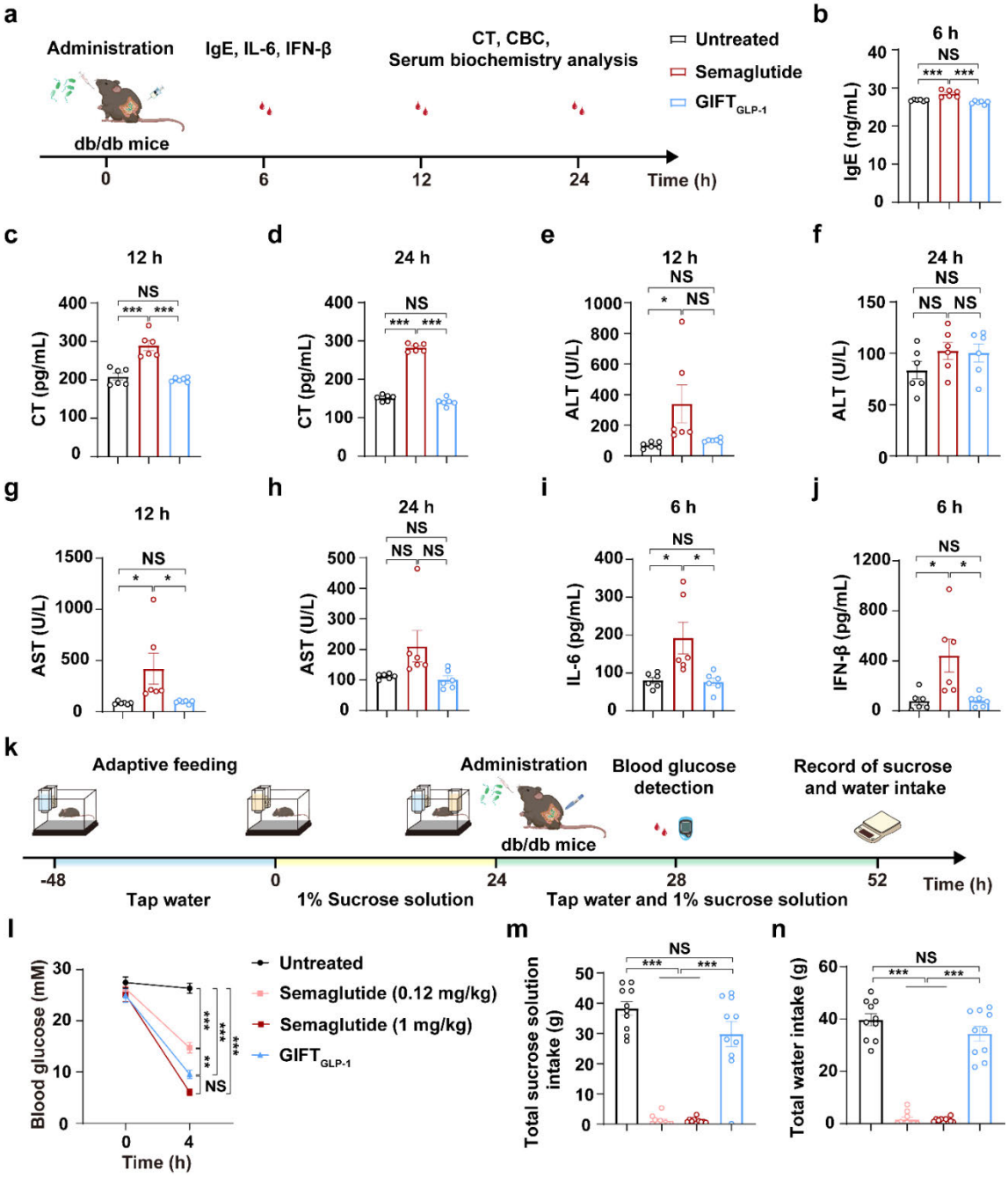
Additionally, we compared the risk of hypoglycemia when both treatments were used in combination with insulin glargine. High doses of semaglutide (1 mg/kg, based on human dosage conversion) and low doses of semaglutide (0.12 mg/kg, referenced from literature) combined with insulin glargine (7 U/kg) each resulted in significant increases in the incidence of hypoglycemia; no increased hypoglycemic events were observed when GIFT was used in combination with insulin glargine (7 U/kg) (new **Supplementary Tables 3-9**).

These findings support the advantages of the GIFT system over a once-weekly injectable intervention in reducing systemic side effects while effectively managing glucose levels. Please see **pages 16-17, lines 446-483** in the revised manuscript and below:

> "We assessed acute inflammatory and hypersensitivity reactions in db/db mice following a single administration of $GIFT_{GLP\text{-}1}$ (10^9 CFU) or semaglutide (1 mg/kg). Blood samples were collected for complete blood count, serum biochemistry, and analysis of immunoglobulin E (IgE), calcitonin, interleukin-6 (IL-6), and interferon beta (IFN-β) levels (**Extended Data Fig. 10a** and **Supplementary Tables 1, 2**). Semaglutide injections triggered several physiological responses not observed in the GIFT group. Specifically, semaglutide-treated mice exhibited significantly elevated IgE levels at 6 hours after dosing, indicating an immediate hypersensitivity reaction (**Extended Data Fig. 10b**). Plasma calcitonin levels also increased at 12 and 24 hours, suggesting potential adverse effects on the thyroid gland (**Extended Data Fig. 10c**, **d**). Elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels at 12 hours post-injection indicated acute hepatic stress, which resolved by 24 hours (**Extended Data Fig. 10e-h**). Serum IL-6 and IFN-β levels were significantly increased at 6 hours after injection, indicating an acute inflammatory response (**Extended Data Fig. 10i**, **j**).
>
> To assess potential adverse responses elicited by $GIFT_{GLP\text{-}1}$ and semaglutide, we conducted a conditioned-taste aversion (CTA) study in db/db mice. CTA is a specialized form of associative learning where animals avoid a taste linked to a detrimental health condition[64]. Mice were given

a 1% sucrose solution, followed by administration of $GIFT_{GLP\text{-}1}$ (10^9 CFU) or semaglutide (0.12 mg/kg or 1 mg/kg) (**Extended Data Fig. 10k**). A two-bottle taste preference test was then performed, offering mice a choice between tap water and the sucrose solution, with consumption monitored over 24 hours. Despite semaglutide-treated mice displaying similar glycemic control levels compared to those treated with $GIFT_{GLP\text{-}1}$ 4 hours after administration (**Extended Data Fig. 10l**), they demonstrated a significantly stronger aversive response to the sucrose solution (**Extended Data Fig. 10m**). Moreover, semaglutide-treated mice consumed significantly less water compared to both untreated mice and those treated with $GIFT_{GLP\text{-}1}$ cells (**Extended Data Fig. 10n**), indicating potential adverse side effects of semaglutide.

Given the complexity of diabetes management, often requiring combination therapies, we assessed the risk of hypoglycemia when $GIFT_{GLP\text{-}1}$ or semaglutide were combined with insulin glargine. db/db mice received insulin glargine alone (7 U/kg), high-dose semaglutide (1 mg/kg, adjusted from human dosages), low-dose semaglutide (0.12 mg/kg, as reported in literature), or $GIFT_{GLP\text{-}1}$ (10^9 CFU). Insulin glargine alone resulted in an approximately 20% risk of hypoglycemia (**Supplementary Table 3**). When combined with insulin glargine, both high and low doses of semaglutide significantly increased hypoglycemia events (**Supplementary Tables 4-7**). In contrast, no increase in hypoglycemia was observed with the combination of $GIFT_{GLP\text{-}1}$ and insulin glargine (**Supplementary Tables 8**, **9**), highlighting the advantage of glucose-responsive drug release. Thus, GIFT cells demonstrate a superior safety profile and fewer behavioral side effects compared to semaglutide in mice."



Response Document Figure 12 (see Extended Data Fig. 10 in the revised manuscript): Comparative analysis of adverse effects between semaglutide injection and oral delivery of GIFT$_{GLP-1}$ in db/db mice. a, Schematic of the timeline for assessing acute adverse effects. db/db mice were divided into three groups: one received oral GIFT$_{GLP-1}$, another was injected with semaglutide, and the third group remained untreated. Blood samples were collected at 6 hours for serum immunoglobulin E (IgE), interleukin-6 (IL-6), and Interferon beta (IFN-β) analysis, and at 12 and 24 hours for complete blood count (CBC), serum biochemical analysis, and calcitonin (CT) measurement. **b**, Serum IgE levels in db/db mice 6 hours after treatment. **c-h**, Serum levels of CT (**c**, **d**), alanine transaminase (ALT) (**e**, **f**), and aspartate transaminase (AST) (**g**, **h**) in db/db mice 12 and 24 hours after treatment. **i**, **j**, Serum levels of IL-6 (**i**) and IFN-β (**j**) in db/db mice 6 hours after treatment. **k**, Schematic of the timeline for the conditioned taste aversion (CTA) experiment. db/db mice were single-housed with *ad libitum* access to chow diet and tap water for 48 hours, followed by access to 1% sucrose for 24 hours.

After acclimation, mice given oral $GIFT_{GLP-1}$ or injected with semaglutide underwent a two-bottle preference test, choosing between tap water and 1% sucrose. Sucrose and water intake were recorded for 24 hours. **l**, Blood glucose levels monitored 4 hours after $GIFT_{GLP-1}$ or semaglutide administration. **m**, Total sucrose intake. **n**, Total water intake. Data in **b-j** and **l-n** are expressed as means ± SEM; $n = 6$ mice in **b-j**, $n = 9$ mice in **l-n**. *P*-values were calculated using one-way ANOVA. NS, not significant; $*P < 0.05$, $**P < 0.01$, $**P < 0.001$.

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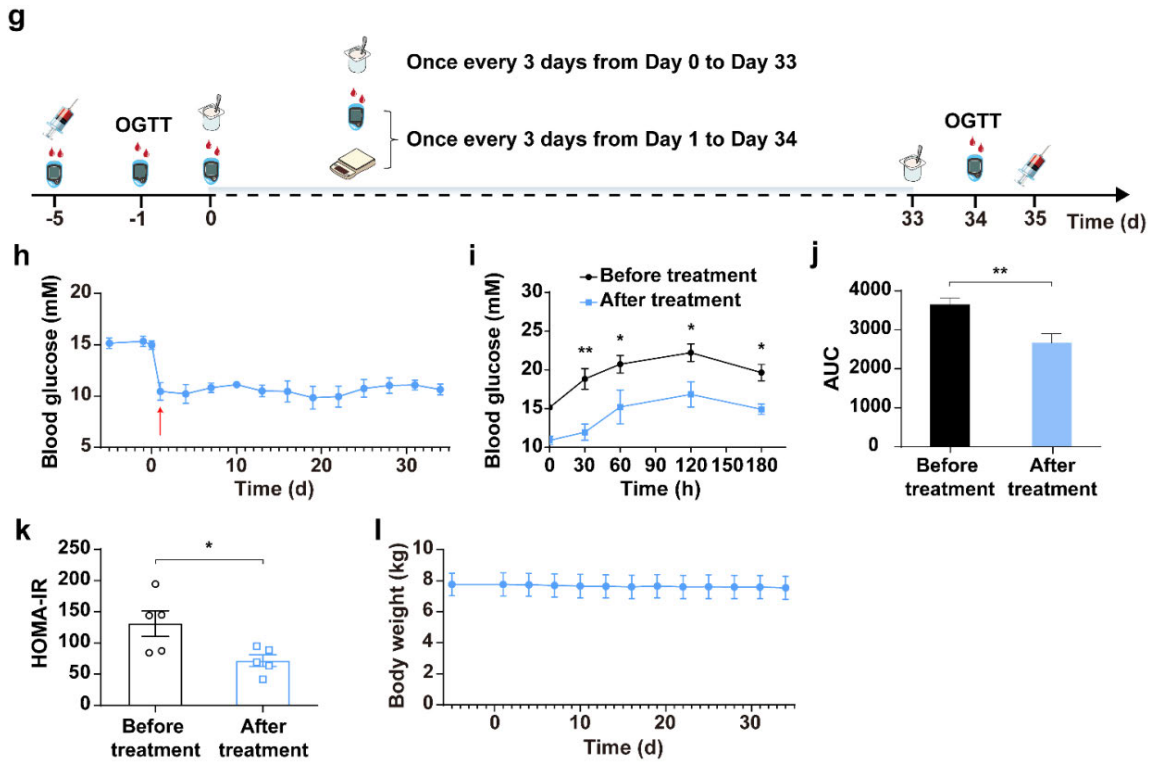
* NHP safety and efficacy data does not extend beyond 3 days. Why is this?

Reply:

Thank you for your comment. Our initial investigations in NHPs were indeed limited to short-term studies, primarily due to constraints related to animal availability and budget considerations. These preliminary trials were designed to assess our live biotherapeutic product's immediate therapeutic potential and basic safety profile. Recognizing the necessity to substantiate our treatment's long-term safety and efficacy, we have since extended our experimental framework. We conducted a five-week study, during which the monkeys were administered $GIFT_{GLP\text{-}1}$ orally every three days (new **Fig. 7g-l**). The results from this extended study, now included in our revised manuscript, demonstrate consistent therapeutic efficacy and a favorable safety profile over the five weeks. Please see **page 17, lines 494-501** in the revised manuscript and below:

> "A five-week study demonstrated that oral administration of $GIFT_{GLP\text{-}1}$ every three days effectively restored glucose homeostasis over an extended period (**Fig. 7g, h**). Further, oral glucose tolerance in the monkeys was significantly improved after five weeks of $GIFT_{GLP\text{-}1}$ treatment (**Fig. 7i**, **j**). Insulin resistance was also notably alleviated (**Fig. 7k**). We also observed a slight improvement in lipid profiles in the diabetic monkeys, as indicated by reductions in serum TG, T-CHO, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) levels following oral administration of $GIFT_{GLP\text{-}1}$ (**Supplementary Tables 10**, **11**)."



Response Document Figure 13 (see Fig. 7g-l in the revised manuscript): g, Time schedule for long-term glycemic control in spontaneous type 2 diabetic monkeys. Five diabetic monkeys were orally administered **GIFT$_{GLP-1}$** (2×10^{11} CFU/kg) every three days from Day 0 to Day 33. Blood glucose and body weight were monitored every three days. Blood was collected for CBC and serum biochemistry analysis on Day -5 and Day 35. **h,** Fasting blood glucose levels. The red arrow represents the time of the first administration. **i**, **j**, Oral glucose tolerance tests (OGTT) were conducted before (Day -1) and after treatment (Day 34). **k**, Insulin resistance was assessed before (Day -5) and after treatment (Day 35). **l,** Body weights of monkeys. Data in **h-l** are expressed as means ± SEM; $n = 5$ monkeys. *P*-values were calculated by *t*-test (**j** and **k**) or one-way ANOVA (**i**). $*P < 0.05$, $**P < 0.01$.

Minor concerns

* Unclear why Fig 1g oscillations does not translate to in vivo db/db model. It would be beneficial to observe the GLP-1 level drop in mice when the blood glucose level is low.

Reply:

Thank you for your constructive guidance. We conducted additional experiments in wild-type and db/db mice to evaluate the dynamic gene expression regulation by GIFT *in vivo*.

First, we administered GIFT$_{Lux}$ cells to wild-type mice via oral gavage and then simulated the ON-OFF-ON dynamics of gene expression by intraperitoneal injection of glucose solution (1g/kg) (new **Fig. 2f-h** and **Supplementary Fig. 2**). Specifically, 30 minutes post-gavage, we injected glucose solution intraperitoneally to elevate the blood glucose levels in the mice, which resulted in the activation of LuxCDABE expression. As the blood glucose levels decreased, the expression of LuxCDABE also diminished. Subsequent glucose injections reactivated the expression of LuxCDABE,

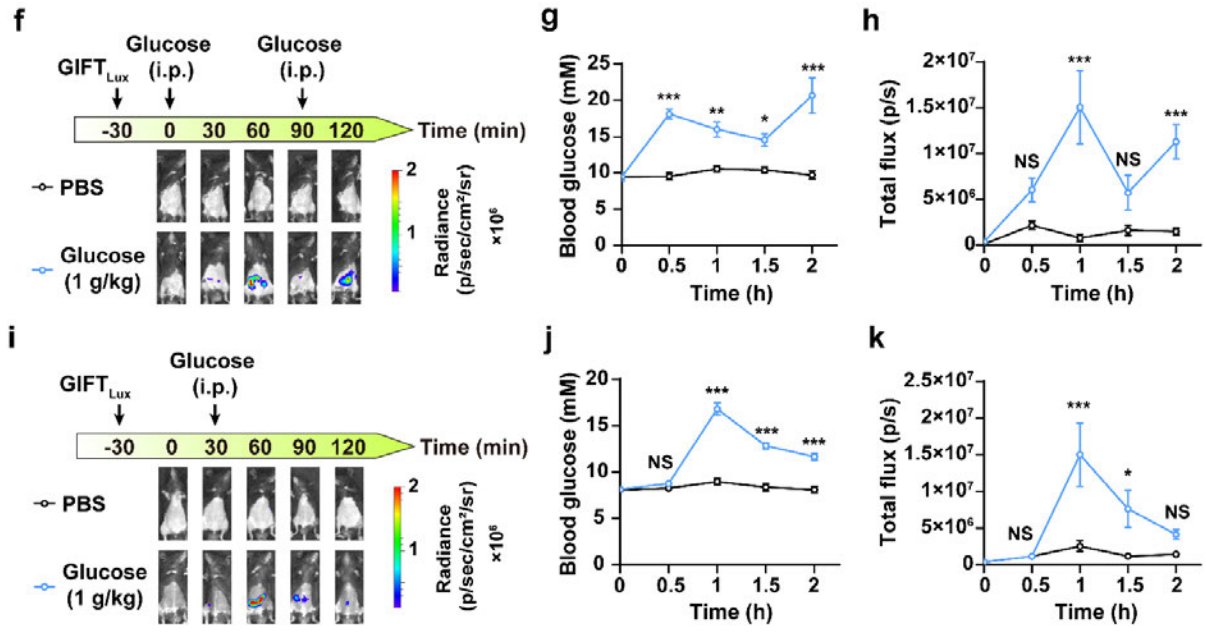
demonstrating the system's ability to respond dynamically to changes in glucose concentration. Similar OFF-ON-OFF dynamics were observed in mice (new **Fig. 2i-k** and **Supplementary Fig. 3**).

Subsequently, we gavaged $GITF_{GLP\text{-}1}$ cells to db/db mice and monitored GLP-1 levels in response to fluctuations in blood glucose. Elevated blood glucose levels induce GLP-1 expression by our $GITF_{GLP\text{-}1}$ cells and decrease as the blood glucose levels fall (new **Fig. 3d, e**). These results illustrate that our GITF cells can control protein expression in response to changes in glucose levels *in vivo*.

These new findings have been added to our revised manuscript. Please see **page 7, lines 194-204,** and **page 9, lines 251-256** in the revised manuscript and below:

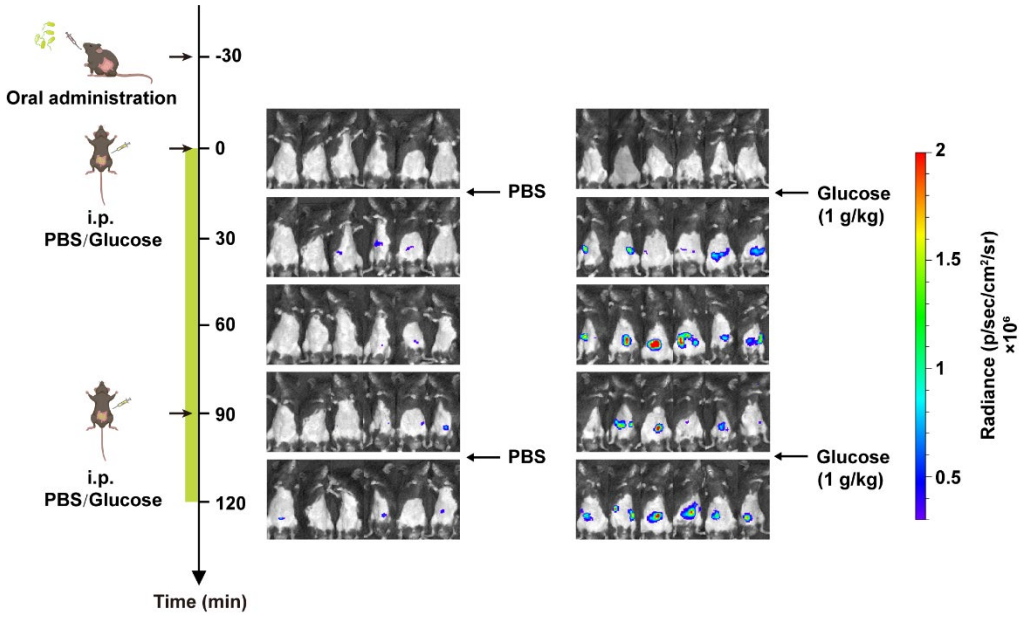
"To explore the ON-OFF-ON gene expression dynamics, we simulated blood glucose fluctuations typically seen in diabetic mice through intraperitoneal glucose injections. Thirty minutes after oral administration of $GIFT_{Lux}$ cells, glucose solution (1 g/kg) was injected intraperitoneally to elevate blood glucose, activating LuxCDABE expression in $GIFT_{Lux}$-treated mice. As blood glucose levels dropped, LuxCDABE expression decreased accordingly. The second glucose injection reactivated LuxCDABE expression, demonstrating the system's ability to dynamically respond to glucose fluctuations (**Fig. 2f-h** and **Supplementary Fig. 2**). Similar OFF-ON-OFF gene expression dynamics were observed in mice orally delivered with $GIFT_{Lux}$ (**Fig. 2i-k** and **Supplementary Fig. 3**). These findings demonstrate that GIFT cells can modulate protein expression in response to changing glucose levels."

"To determine whether the improved glycemic control in $GIFT_{GLP\text{-}1}$-treated db/db mice is due to hyperglycemic-triggered GLP-1 expression, we investigated GLP-1 dynamics following oral administration of $GIFT_{GLP\text{-}1}$. After administering $GIFT_{GLP\text{-}1}$ cells to db/db mice, we monitored blood glucose and serum GLP-1 levels hourly. GLP-1 expression was effectively induced by elevated blood glucose, peaking between 2 to 4 hours post-administration, and declining as glucose levels normalized (**Fig. 3d, e**)."

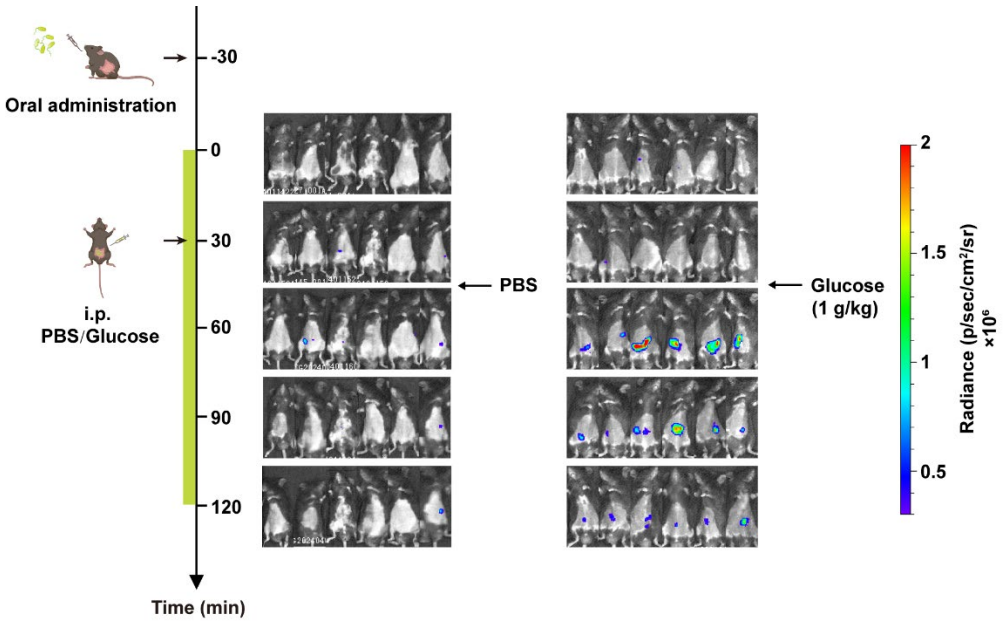


Response Document Figure 14 (see Fig. 2f-k in the revised manuscript): GIFT-mediated gene expression

kinetics after oral delivery to mice. f-h, GIFT-mediated gene expression kinetics (ON-OFF-ON) in wild-type mice. Mice were orally administered $GIFT_{Lux}$ cells (10^9 CFU) at -30 min, and intraperitoneally injected (i.p.) with PBS or glucose (1 g/kg) at 0 and 90 min. Blood glucose (**g**) and bioluminescence signals (**f**, **h**) were recorded every 30 min. **i-k**, GIFT-mediated gene expression kinetics (OFF-ON-OFF) in wild-type mice. Mice received $GIFT_{Lux}$ cells (10^9 CFU) at -30 min, and intraperitoneally injected (i.p.) with PBS or glucose (1 g/kg) at 30 min. Blood glucose (**j**) and bioluminescence signals (**i**, **k**) were recorded every 30 min. Data in **g**, **h**, **j**, and **k** are expressed as means ± SEM; $n = 6$ mice. *P*-values were calculated by two-way ANOVA. NS, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

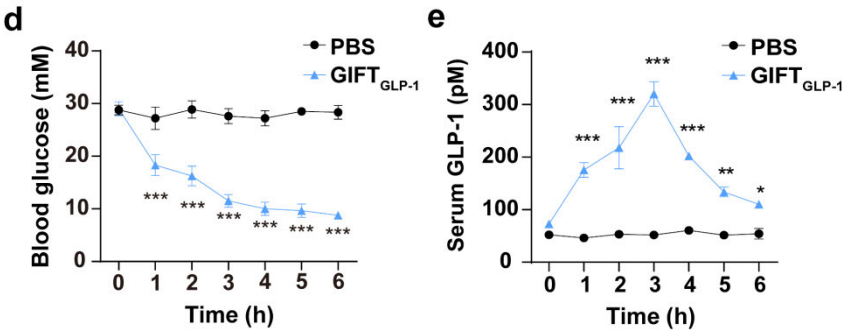


Response Document Figure 15 (see Supplementary Fig. 2 in the revised manuscript): GIFT-mediated gene expression kinetics (ON-OFF-ON) in wild-type mice. After a 4-hour fast, wild-type mice were orally administered $GIFT_{Lux}$ cells (10^9 CFU) at -30 min, followed by intraperitoneal injection (i.p.) of PBS or glucose (1 g/kg) at 0 and 90 min. Imaging was performed every 30 min using an *in vivo* imaging system.



Response Document Figure 16 (see Supplementary Fig. 3 in the revised manuscript): GIFT-mediated gene expression kinetics (OFF-ON-OFF) in wild-type mice. After a 4-hour fast, wild-type mice were orally

administered GIFT$_{Lux}$ cells (10^9 CFU) at -30 min, followed by intraperitoneal injection (i.p.) of PBS or glucose (1 g/kg) at 30 min. Imaging was performed every 30 min using an *in vivo* imaging system.



Response Document Figure 17 (see new Fig. 3d, e in the revised manuscript): Changes in blood glucose and serum GLP-1 levels in db/db mice following oral administration of GIFT$_{GLP-1}$ cells. GIFT$_{GLP-1}$ cells (10^9 CFU) were administered via gavage, and blood glucose (**d**) and serum GLP-1 levels (**e**) were monitored over 6 hours. Data are expressed as means ± SEM; $n = 5$ mice. *P*-values were calculated using two-way ANOVA. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

* Since the GIFT_GLP-1 senses the glucose in the intestine, how would the glucose from food that goes to intestine affect the sensor dynamics in vivo?

Reply:

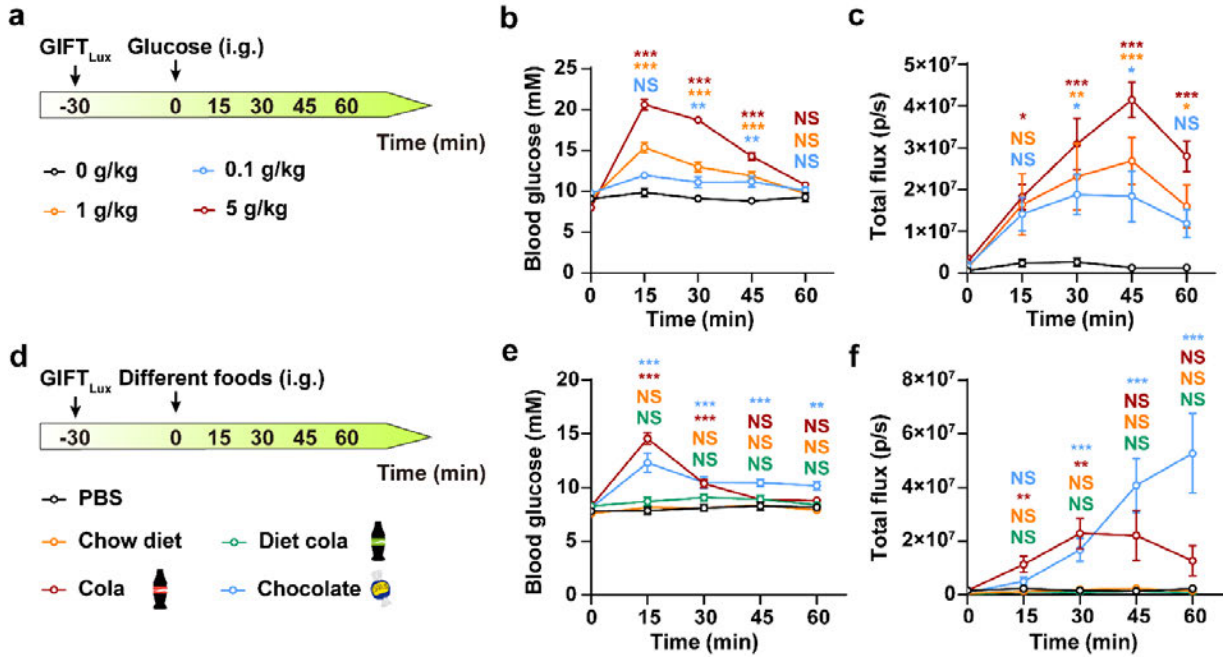
Thank you for your constructive guidance.

1. We conducted a series of new *in vivo* experiments to investigate how glucose from food affects sensor dynamics. Initially, wild-type mice were orally administered varying glucose concentrations 30 minutes after receiving GIFT$_{Lux}$ cells (10^9 CFU). Blood glucose levels and bioluminescence signal intensities were quantified every 15 minutes, which showed that oral glucose induced gene expression, with expression levels increasing alongside glucose concentration (new **Extended Data Fig. 9a-c** and **Supplementary Fig. 5**). Subsequently, we administered different types of sugary food to wild-type mice, high-fat diet (HFD)-induced obese mice, and db/db mice to evaluate the effects on our sensor dynamics. Briefly, the results demonstrate that the glucose content in food influences the dynamics of our sensor system. For instance, when dietary glucose was introduced into the gastrointestinal tract by consuming high-sugar foods like cola (containing 11.2 g carbohydrates per 100 mL) and chocolate (containing 43 g carbohydrates per 100 g), we observed an activation of gene expression in our GIFT cells. In contrast, low-sugar foods such as chow diet and diet cola did not significantly increase gene expression (new **Fig. 5a-f, Extended Data Fig. 9d-f**, **Supplementary Fig. 6-8**). These findings support that the sensor is capable of effectively detecting and responding to postprandial glucose fluctuations. These findings have been incorporated into our revised manuscript. Please see **page 13, lines 360-377** in the revised manuscript and below:

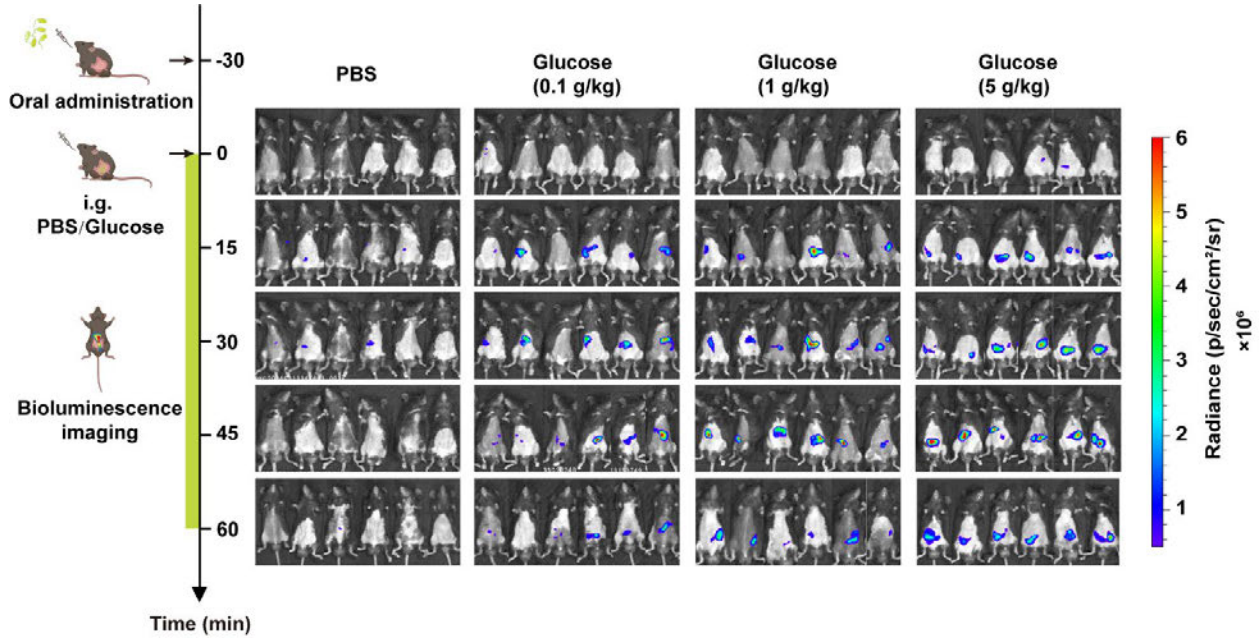
> "GIFT cells sense glucose in the intestine, we assessed the impact of dietary glucose on their sensor dynamics *in vivo*. Wild-type mice were orally administered GIFT$_{Lux}$ cells (10^9 CFU), followed by intragastric delivery of varying glucose concentrations (0, 0.1, 1, and 5 g/kg) 30 min

later. We monitored blood glucose levels and bioluminescence signal intensities, and observed a significant, dose-dependent induction of gene expression corresponding to the administered glucose concentrations (**Extended Data Fig. 9a-c** and **Supplementary Fig. 5**).

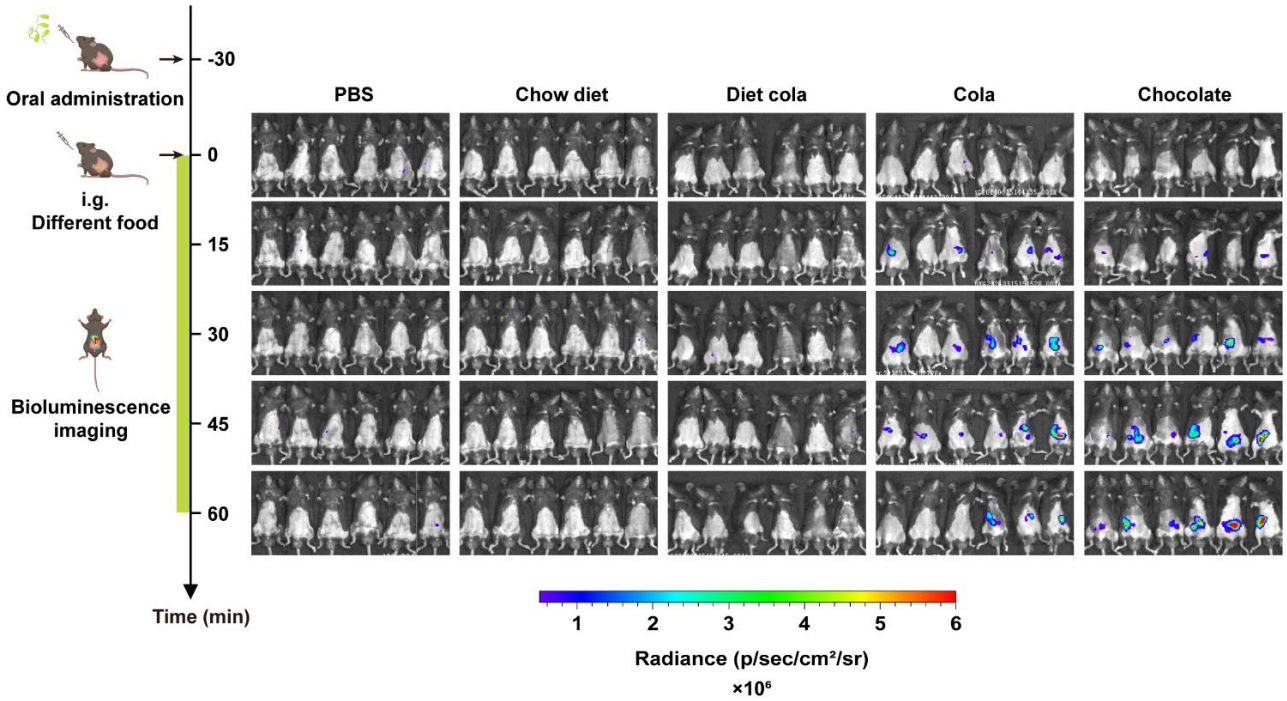
We also assessed the effect of various foods and beverages with different glucose content on sensor responsiveness. Wild-type mice administered $GIFT_{Lux}$ cells (10^9 CFU) were fed low-sugar items like a chow diet and diet cola, or high-sugar items such as cola (11.2 g carbohydrates per 100 mL) and chocolate (43 g carbohydrates per 100 g). High-sugar foods triggered rapid blood glucose spikes, leading to a corresponding increase in gene expression in $GIFT_{Lux}$ cells (**Extended Data Fig. 9d-f** and **Supplementary Fig. 6**), while low-sugar foods did not induce a significant response. These results confirm that the sensor effectively detects and responds to postprandial glucose fluctuations. Further, in high-fat diet-induced obese (DIO) mice and db/db mice, high-sugar foods like cola and chocolate significantly enhanced gene expression in GIFT cells (**Fig. 5a-f** and **Supplementary Fig. 7, 8**)."



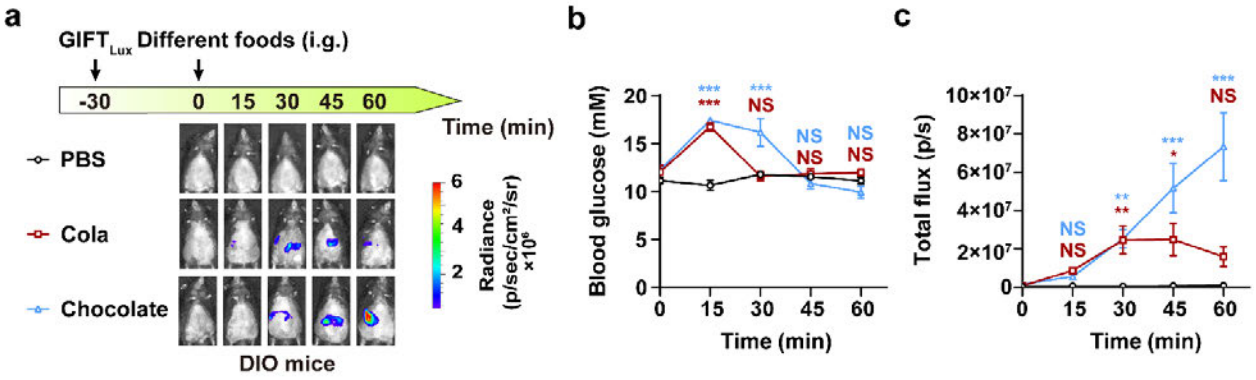
Response Document Figure 18 (see Extended Data Fig. 9a-f in the revised manuscript): Impact of oral administration of varying concentrations of glucose and different sugary foods on blood glucose levels and GIFT-mediated gene expression dynamics in wild-type mice. a, Wild-type mice were orally administered $GIFT_{Lux}$ cells (10^9 CFU). Thirty minutes post-gavage, the mice were administered intragastrically (i.g.) with varying glucose concentrations (0, 0.1, 1, and 5 g/kg). Blood glucose levels (**b**) and bioluminescence signal intensity (**c**) were monitored every 15 min. **d**, Wild-type mice were orally administered $GIFT_{Lux}$ cells (10^9 CFU). Thirty minutes post-gavage, the mice were given either a chow diet or administered intragastrically (i.g.) with 10 μL/g of PBS, diet cola, cola (containing 10.6 g carbohydrates per 100 mL), or chocolate (containing 43 g carbohydrates per 100 g). Blood glucose levels (**e**) and bioluminescence signal intensity (**f**) were monitored every 15 min. Data in **b**, **c**, **e**, and **f** are expressed as means ± SEM; $n = 6$ mice. *P*-values were calculated by two-way ANOVA. NS, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.



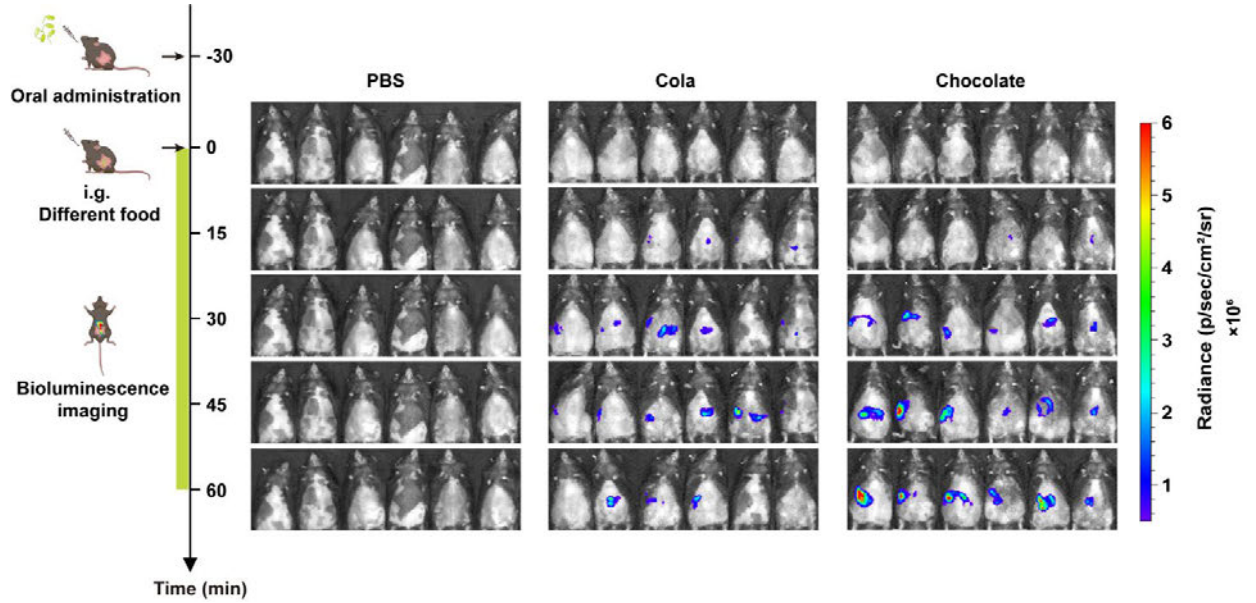
Response Document Figure 19 (see Supplementary Fig. 5 in the revised manuscript): Effects of oral administration of varying concentrations of glucose on blood glucose levels and GIFT-mediated gene expression dynamics in wild-type mice. Wild-type mice were orally administered $GIFT_{Lux}$ cells (10^9 CFU). Thirty minutes post-gavage, the mice were intragastric (i.g.) administration of glucose at concentrations of 0, 0.1, 1, and 5 g/kg. Imaging was performed every 15 min using an *in vivo* imaging system.



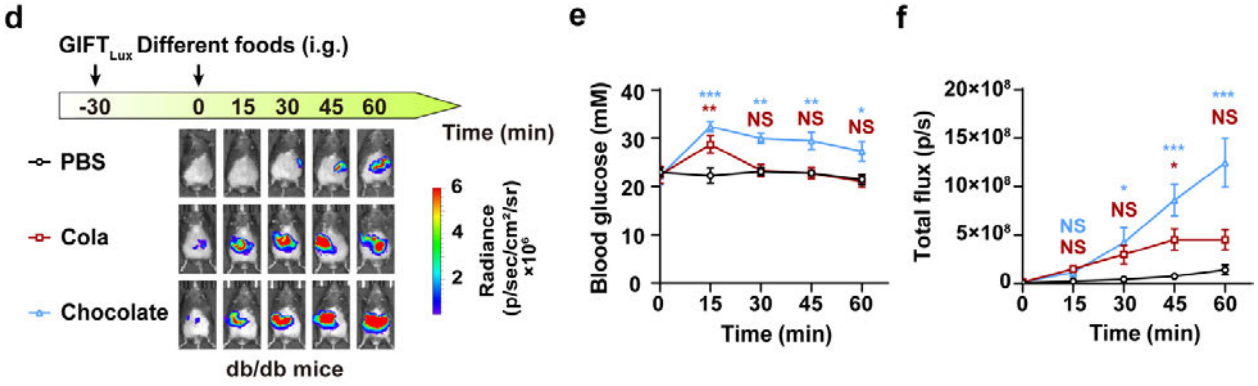
Response Document Figure 20 (see Supplementary Fig. 6 in the revised manuscript): Effects of oral administration of different sugary foods on blood glucose levels and GIFT-mediated gene expression dynamics in wild-type mice. Wild-type mice were orally administered $GIFT_{Lux}$ cells (10^9 CFU). Thirty minutes post-gavage, the mice were either given a chow diet or administered intragastrically (i.g.) with PBS, diet cola, cola, or chocolate. Imaging was performed every 15 min using an *in vivo* imaging system.



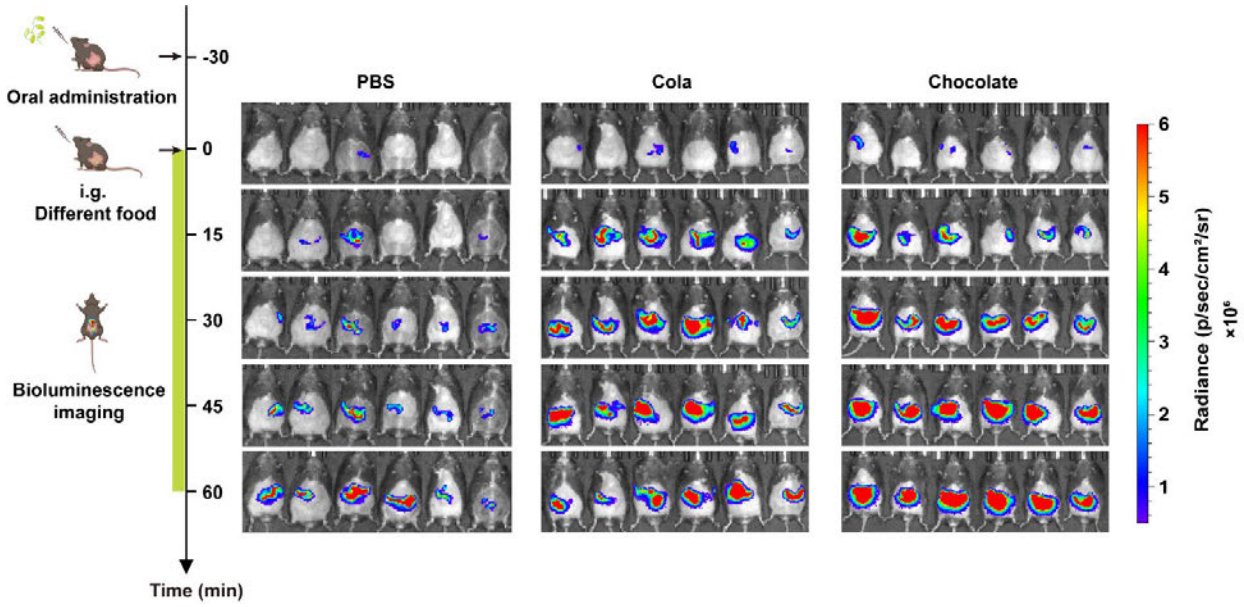
Response Document Figure 21 (see Fig. 5a-c in the revised manuscript): Effects of oral administration of different sugary foods on blood glucose levels and GIFT-mediated gene expression dynamics in DIO mice. Mice were orally administered GIFT$_{Lux}$ cells (10^9 CFU), and 30 min later, they received an intragastric (i.g.) administration of PBS, cola, or chocolate. Blood glucose levels (**b**) and bioluminescence signal intensity (**a**, **c**) were monitored every 15 minutes. Data in panels **b, c** are expressed as means ± SEM; n = 6 mice. P-values were calculated using two-way ANOVA. NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001.



Response Document Figure 22 (see Supplementary Fig. 7 in the revised manuscript): Effects of oral administration of different sugary foods on GIFT-mediated gene expression dynamics in DIO mice. DIO mice were orally administered GIFT$_{Lux}$ cells (10^9 CFU). Thirty minutes post-gavage, the mice were administered intragastrically (i.g.) with PBS, cola, or chocolate. Imaging was performed every 15 min using an *in vivo* imaging system.



Response Document Figure 23 (see Fig. 5d-f in the revised manuscript): Effects of oral administration of different sugary foods on blood glucose levels and GIFT-mediated gene expression dynamics in db/db mice. Mice were orally administered GIFT$_{Lux}$ cells (10^9 CFU), and 30 min later, they received an intragastric (i.g.) administration of PBS, cola, or chocolate. Blood glucose levels (**e**) and bioluminescence signal intensity (**d**, **f**) were monitored every 15 min. Data in panels **e-f** are expressed as means ± SEM; n = 6 mice. P-values were calculated using two-way ANOVA. NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001.

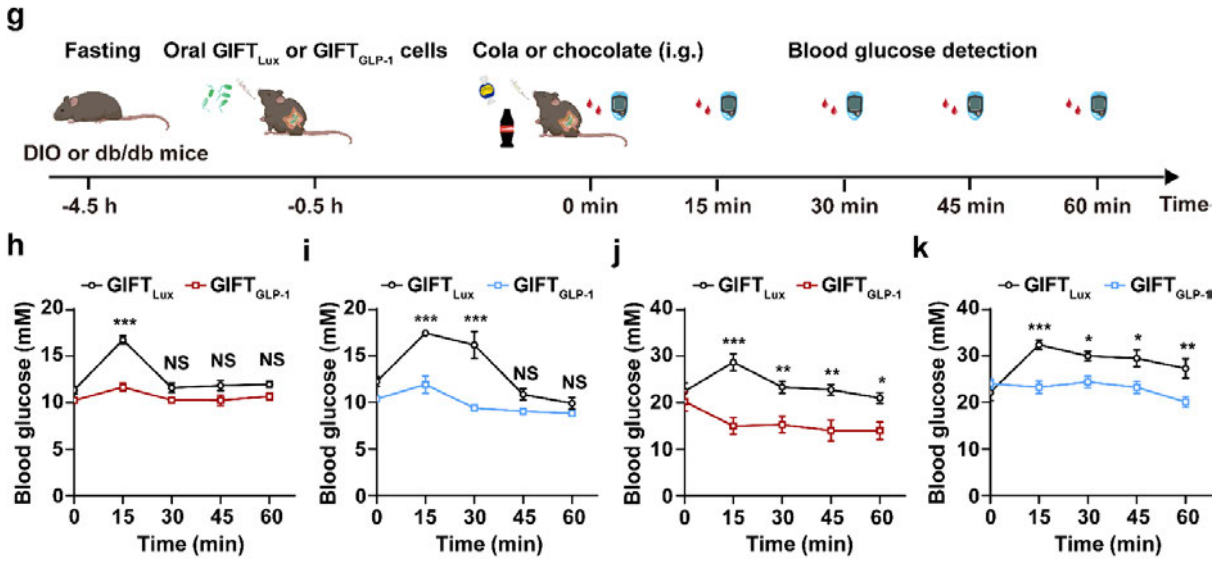


Response Document Figure 24 (see Supplementary Fig. 8 in the revised manuscript): Effects of oral administration of different sugary foods on GIFT-mediated gene expression dynamics in db/db mice. db/db mice were orally administered GIFT$_{Lux}$ cells (10^9 CFU). Thirty minutes post-gavage, the mice were administered intragastrically (i.g.) with PBS, cola, or chocolate. Imaging was performed every 15 min using an *in vivo* imaging system.

2. We also assessed the impacts of high-sugar foods by conducting additional experiments in which we administered cola or chocolate to DIO or db/db mice receiving either GIFT$_{Lux}$ or GIFT$_{GLP\text{-}1}$ cells (10^9 CFU) and then monitored blood glucose levels at 15-minute intervals. We found that the DIO and

db/db mice that received GIFT$_{GLP-1}$ cells exhibited significantly reduced extents of postprandial blood glucose rise, indicating that the transient increase in intestinal glucose after eating can activate GIFT cells to produce GLP-1, thereby helping to moderate the postprandial blood glucose rise (new **Figure 5g-k**). These findings have been added to our revised manuscript. Please see **page 14, lines 383-388** in the revised manuscript and below:

> "We then administered GIFT$_{Lux}$ or GIFT$_{GLP-1}$ cells to DIO mice and db/db mice. Thirty minutes post-gavage, these mice were fed cola and chocolate to induce postprandial glucose spikes. Mice treated with GIFT$_{GLP-1}$ cells showed significantly lower blood glucose increase compared to those treated with GIFT$_{Lux}$ cells (**Fig. 5g-k**), indicating that GIFT$_{GLP-1}$ cells, activated by transient glucose elevations, effectively produce GLP-1 to moderate glucose spikes."



Response Document Figure 25 (see Fig. 5g-k in the revised manuscript): GIFT-mediated postprandial glycemic control in high-fat diet-induced obese (DIO) mice and db/db mice. g, Time schedule for oral administration of GIFT$_{GLP-1}$ cells for postprandial glycemic control in DIO and db/db mice. After a 4-hour fasting period, mice were orally administered either GIFT$_{Lux}$ or GIFT$_{GLP-1}$ cells (10^9 CFU) and received cola or chocolate 30 min later. Blood glucose levels were monitored every 15 minutes. **h**, **i**, Blood glucose levels in DIO mice following intragastric administration of cola (**h**) or chocolate (**i**). **j**, **k**, Same as for **h**, **i**, but in db/db mice. Data in panels **h-k** are expressed as means ± SEM; $n = 6$ mice. *P*-values were calculated using two-way ANOVA. NS, not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

* In Supplementary Figure 7, the production of GLP-1 by GIFT is quantified from its supernatant. How is the GLP-1 released from EcN?

Reply:

Thank you for your question. In our design, the GLP-1 is secreted by the GIFT cells via an engineered expression system where the GLP-1 gene is fused to a signal peptide sequence at the N-terminus. We tested several secretion signal peptides, including commonly used ones like PelB, PhoA, OmpA, OmpT, and LamB, for protein secretion in our engineered bacteria. The OmpT and LamB signal

peptides demonstrated the best secretion outcomes. This information has been provided in our manuscript's Materials and Methods section and the plasmid construction table.

Again, we express our sincere gratitude for the helpful guidance in improving our study and manuscript. Many thanks!

Referee #2 (Remarks to the Author):

In this study, Guan and colleagues developed an engineered oral glucose-sensing and self-feedback probiotic (GIFT) living drug for glucose control. They created sensor cells that temporarily reside in the intestine and regulate the expression of GLP1 in response to glucose. The authors demonstrated a four-fold increase in plasma GLP-1 levels in db/db mice and NHPs, resulting in reduced plasma glucose levels, food intake, and body weight. The authors demonstrate increased energy expenditure, reduced levels of liver fat and inflammation, and reduced kidney complications. Based on these findings, the authors conclude that they have developed a novel method for precise therapeutic GLP1 dosing, which can be applied in the long term to promote adherence.

This work presents a new approach to treating diabetes and potentially other metabolic diseases, such as obesity and MALFD, which is of great importance. However, the reviewer has outlined several points that remain unclear and need clarification to understand the mode of action of this new approach.

Reply:

We appreciate this reviewer's positive comments and constructive guidance.

Major

- The figure numbers are not in the same order as they appear in the text, which sometimes makes it difficult to follow the flow of the experiments. I would recommend that the authors streamline the figure numbers in the text with the progressive narrative of the manuscript.

Reply:

Thank you for your valuable feedback. We have carefully reviewed the manuscript and adjusted the figure numbers to ensure they follow the experiments presented in the text.

- The authors demonstrate glucose-dependent sfGFP expression in cell culture through GIFT. However, the glucose concentration in the gut lumen of mice and NHPs (Figures 2, 5), where the GIFT cells temporarily reside, is not provided. This is a critical point since it is unclear whether high plasma glucose levels correspond to high intestinal glucose levels or whether glucose levels in the gut rather reflect glucose intake. For instance, can the authors demonstrate the response of a db/db mouse to a low-carb/low-calorie diet with and without GIFT in terms of GLP1, plasma glucose and insulin levels, and body weight? Does their approach also work in diet induced obese mice?

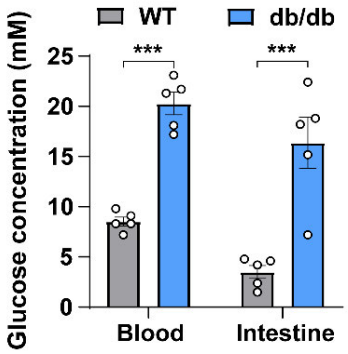
Reply:

Thank you for your valuable feedback.

1. To elucidate the glucose concentrations in the gut lumen, we have completed new experiments examining wild-type and db/db mice (8-week-old). Initially, we measured their fasting blood glucose levels. Afterward, the mice were euthanized, their intestines were harvested, and the mucosal lining of the intestines was then collected. Glucose content was measured using a commercial glucose assay kit (ab65333, Abcam). We found that although the glucose concentrations within the intestine are lower than those in the blood, intestinal glucose concentrations in db/db mice were significantly higher than in wild-type mice, approximately 15 mM (**Extended Data Fig. 5a**); these data corroborate recent

findings from research utilizing an ingestible biosensing device, which demonstrated a positive correlation between intestinal and blood glucose concentrations (De la Paz et al., *Nat Commun*, 2022). This observation supports the hypothesis that GIFT cells residing in the gut of diabetic mice can be activated by elevated glucose levels, thereby releasing GLP-1 to reduce blood glucose levels effectively. These results have been incorporated into the revised manuscript. Please see **page 8, lines 206-212** in the revised manuscript and below:

> "We measured fasting blood glucose and intestinal glucose concentrations in the gut lumen of both wild-type and leptin receptor-deficient (db/db) mice. Fasting blood glucose was below 10 mM in wild-type mice and over 16.6 mM in db/db mice. Although intestinal glucose concentrations were lower than blood levels, they were significantly higher in db/db mice, reaching approximately 15 mM (**Extended Data Fig. 5a**). These elevated intestinal glucose levels in diabetic mice suggest that they are sufficient to activate GIFT."



Response Document Figure 26 (see Extended Data Fig. 5a in the revised manuscript): Measurement of intestinal glucose concentrations in wild-type and db/db mice. Following a 4-hour fasting period, the blood glucose levels of the mice were measured. The mice were then euthanized, and their intestines were harvested; the mucosal lining of the intestines was collected. Glucose content was measured using a commercial glucose assay kit. Data are expressed as means ± SEM; $n = 5$ mice. *P*-values were calculated by two-way ANOVA. ***$P < 0.001$.

2. In response to your guidance, we conducted a series of new experiments to assess the efficacy of our GIFT cells across different models of diabetes and obesity. First, we conducted a study with db/db mice subjected to a low-carbohydrate diet (comprising 25% protein, 65% fat, and 10% carbohydrates) (Long et al., *Mol Metab*, 2023; Lin et al., *J Nutr Biochem*, 2022). We monitored parameters including blood glucose, GLP-1 levels, insulin levels, body weight, fat mass, serum triglycerides (TG), and serum total cholesterol (T-CHO) for a total of 30 days. With this diet, $GIFT_{GLP\text{-}1}$ cells remained responsive to elevated blood glucose levels, effectively maintaining blood glucose homeostasis and contributing to weight and fat mass reduction (new **Fig. 6a-h**).

We also evaluated GIFT cells in high-fat diet-induced obese (DIO) mice (new **Fig. 6i-p**). These mice do not display significantly elevated blood glucose levels, which are required for GIFT cell activation. However, long-term oral administration of $GIFT_{GLP\text{-}1}$ cells resulted in a 5% body weight reduction in these mice (new **Fig. 6n**). Additionally, insulin resistance, fat mass, and serum TG and T-

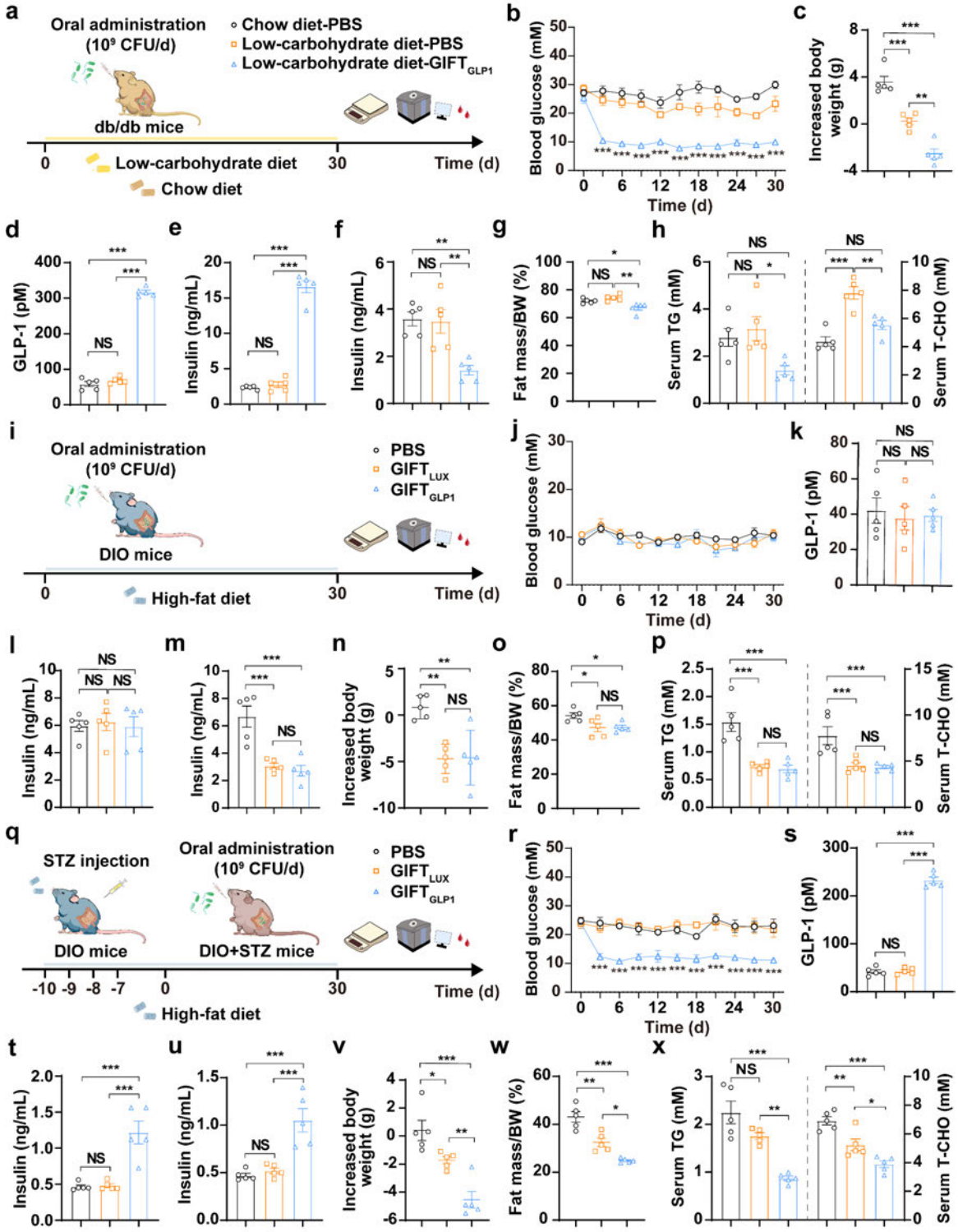
CHO levels were significantly lower in $GIFT_{GLP-1}$-treated mice compared to the PBS control group (new **Fig. 6m-p**). Similarly, mice treated with $GIFT_{Lux}$ demonstrated comparable reductions in insulin resistance, body weight, and fat mass. These effects may be attributed to the probiotic properties of the GIFT cells. Previous studies have demonstrated that a high-fat diet can disrupt the gut microbiome, and EcN can modify the gut microbiome (Bisanz et al., *Cell Host Microbe*, 2019; Shi et al., *Front Microbiol*, 2023). Alterations in the gut microbiome have been linked to improvements in insulin sensitivity, lipid metabolism, and inflammation (Cani et al., *Diabetes*, 2008; Velloso et al., *Endocr Rev*, 2015). However, the exact mechanisms behind the weight loss and potential fat reduction effects require further detailed investigation.

To explore the effectiveness of GIFT cells in the nongenetic type 2 diabetes model (Kim et al., *Exp Mol Med*, 2023; Gilbert et al., *Exp Diabetes Res*, 2011), we treated DIO mice with streptozotocin to induce diabetes. This approach allowed us to assess the efficacy of $GIFT_{GLP-1}$ cells in a model that mimics the metabolic characteristics of type 2 diabetes mellitus in humans. Administration of $GIFT_{GLP-1}$ cells in these mice resulted in a significant reduction in blood glucose levels, achieving a 10% decrease; control mice given $GIFT_{Lux}$ cells did not exhibit a similar reduction (new **Fig. 6q-x**). These findings support using $GIFT_{GLP-1}$ cells to actively manage glucose levels in diabetic animals.

These results have been incorporated into the revised manuscript. Please see **pages 14-15, lines 392-427** in the revised manuscript and below:

> "We evaluated the therapeutic potential of GIFT cells across multiple mouse models representing distinct forms of diabetes and obesity. First, we examined the responsiveness of $GIFT_{GLP-1}$ cells in db/db mice subjected to a restrictive low-carbohydrate diet (25% protein, 65% fat, and 10% carbohydrates)[57]. Mice received a daily oral dose of 10^9 CFU $GIFT_{GLP-1}$ cells, and fasting blood glucose levels and body weight were monitored over 30 days (**Fig. 6a**). The low-carbohydrate diet modestly reduced blood glucose levels and significantly decreased body weight compared to a standard chow diet (22.9% protein, 11.1% fat, and 66% carbohydrates) (**Fig. 6b**, **c**). Daily oral administration of $GIFT_{GLP-1}$ cells induced GLP-1 expression, increased insulin levels, lowered blood glucose, and resulted in greater body weight reduction (**Fig. 6b-e**). Moreover, mice treated with $GIFT_{GLP-1}$ cells exhibited decreased insulin resistance, and reductions in fat mass, serum TG, and serum T-CHO compared to PBS controls (**Fig. 6f-h**). These findings indicate that $GIFT_{GLP-1}$ cells are activated by elevated glucose levels in db/db mice, contributing to blood glucose control and body weight management.
>
> We next evaluated the performance of GIFT cells in a DIO mouse model, where mice were given a daily oral dose of 10^9 CFU $GIFT_{GLP-1}$ or $GIFT_{Lux}$ (control). Fasting blood glucose levels were monitored every three days (**Fig. 6i**). Throughout the 30-day experimental period, neither $GIFT_{Lux}$ nor $GIFT_{GLP-1}$ cells significantly altered blood glucose levels (**Fig. 6j**), as GLP-1 and insulin levels were not significantly induced by the GIFT cells (**Fig. 6j-l**). This is consistent with the minimal glucose elevation typically seen in DIO mice[58], which is insufficient to activate the GIFT system. However, long-term oral administration of $GIFT_{GLP-1}$ cells (30 days) resulted in a significant reduction in insulin resistance, body weight, and fat mass (**Fig. 6m-o**). In addition, serum TG and T-CHO were significantly lower in $GIFT_{GLP-1}$-treated mice compared to the PBS control group (**Fig. 6p**). Similar reductions in insulin resistance, body weight, fat mass, serum TG, and T-CHO were also observed in $GIFT_{Lux}$-treated control mice (**Fig. 6m-p**), likely due to the probiotic effects of modulating the gut microbiome that a high-fat diet had disrupted[59, 60].

To further assess the efficacy of GIFT cells in a model that mimics the metabolic characteristics of type 2 diabetes mellitus in humans, we induced diabetes in DIO mice using streptozotocin, creating a nongenetic type 2 diabetes model[61] (**Fig. 6q**). These mice received a daily oral dose of 10^9 CFU $GIFT_{GLP-1}$ or $GIFT_{Lux}$ cells for 30 days. $GIFT_{GLP-1}$ treatment successfully restored glucose homeostasis by increasing GLP-1 and insulin levels compared to PBS and $GIFT_{Lux}$ controls (**Fig. 6r-u**). Moreover, $GIFT_{GLP-1}$-treated mice exhibited significantly lower body weight, fat mass, serum TG, and T-CHO levels (**Fig. 6v-x**). These findings support the potential of $GIFT_{GLP-1}$ cells for managing glucose levels in diabetic models."



Response Document Figure 27 (see Fig. 6 in the revised manuscript): GIFT-mediated diabetes and obesity management in different mouse models. a, Schematic timeline for GIFT-mediated long-term glycemic control in db/db mice on a low-carbohydrate diet. Mice received daily oral gavage of PBS or $GIFT_{GLP\text{-}1}$ cells (10^9 CFU) for 30 days. As a control, db/db mice on a regular chow diet received a daily oral gavage of PBS for the same duration. Serum GLP-1 and plasma insulin were measured 2 hours after the first gavage. Fasting blood glucose was monitored every three days, and lipid profiles and plasma insulin were assessed 24 hours after the final gavage. **b-h,** Data for db/db mice: fasting blood glucose (**b**), body weight (**c**), serum GLP-1 (**d**), plasma insulin levels 2 hours after the first gavage (**e**) and 24 hours after the final gavage (**f**), fat mass (**g**), serum TG and T-CHO levels (**h**). **i**, Schematic timeline for GIFT-mediated long-term glycemic control in DIO mice. Mice were treated with PBS, $GIFT_{Lux}$, or $GIFT_{GLP\text{-}1}$ cells (10^9 CFU) for 30 days. Serum GLP-1 and plasma insulin were measured 2 hours after the first gavage, and fasting blood glucose was monitored every three days. Lipid profiles and plasma insulin were evaluated 24 hours after the final gavage. **j-p,** Data for DIO mice: fasting blood glucose (**j**), serum GLP-1 (**k**), plasma insulin levels 2 hours after the first gavage (**l**) and 24 hours after the final gavage (**m**), body weight (**n**), fat mass (**o**), and serum TG and T-CHO levels (**p**). **q**, Schematic timeline for GIFT-mediated glycemic control in a nongenetic type 2 diabetic mouse model. Diabetes was induced in high-fat diet-fed mice using streptozotocin. Mice were treated with PBS, $GIFT_{Lux}$, or $GIFT_{GLP\text{-}1}$ cells (10^9 CFU) for 30 days. Serum GLP-1 and plasma insulin were measured 2 hours after the first gavage, fasting blood glucose was monitored every three days, and lipid profiles and plasma insulin were assessed 24 hours after the final gavage. **r-x,** Data for nongenetic type 2 diabetic mice: fasting blood glucose (**r**)**,** serum GLP-1 (**s**), plasma insulin levels 2 hours after the first gavage (**t**) and 24 hours after the final gavage (**u**), body weight (**v**), and serum TG and T-CHO levels (**x**). Data in **b-h**, **j-p**, and **r-x** are expressed as means ± SEM; $n = 5$ mice. *P*-values were calculated by one-way ANOVA. NS, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

References:

De la Paz E, Maganti NH, Trifonov A, Jeerapan I, Mahato K, Yin L, Sonsa-Ard T, Ma N, Jung W, Burns R, Zarrinpar A, Wang J, Mercier PP. A self-powered ingestible wireless biosensing system for real-time in situ monitoring of gastrointestinal tract metabolites. Nat Commun. 2022.13(1):7405.

Long F, Bhatti MR, Kellenberger A, et al. A low-carbohydrate diet induces hepatic insulin resistance and metabolic associated fatty liver disease in mice. Mol Metab. 2023.69:101675.

Lin DT, Kao NJ, Cross TL, Lee WJ, Lin SH. Effects of ketogenic diet on cognitive functions of mice fed high-fat-high-cholesterol diet. J Nutr Biochem. 2022.104:108974.

Gilbert ER, Fu Z, Liu D. Development of a nongenetic mouse model of type 2 diabetes. Exp Diabetes Res. 2011.2011:416254.

Kim E, Seo SH, Hwang Y, et al. Inhibiting the cytosolic function of CXXC5 accelerates diabetic wound healing by enhancing angiogenesis and skin repair. Exp Mol Med. 2023.55(8):1770-1782.

Bisanz JE, Upadhyay V, Turnbaugh JA, Ly K, Turnbaugh PJ. Meta-analysis reveals reproducible gut microbiome alterations in response to a high-fat diet. Cell Host Microbe. 2019.26(2):265-272.

Shi J, Ma D, Gao S, Long F, Wang X, Pu X, Cannon RD, Han TL. Probiotic Escherichia coli Nissle 1917-derived outer membrane vesicles modulate the intestinal microbiome and host gut-liver metabolome in obese and diabetic mice. Front Microbiol. 2023.14:1219763.

Cani P, Bibiloni R, Knauf C, Waget A, Neyrinck A, Delzenne N, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet–induced obesity and diabetes in mice. Diabetes.

2008.57:1470-1481.
Velloso L, Folli F, Saad, M. TLR4 at the Crossroads of Nutrients, Gut Microbiota, and Metabolic Inflammation. Endocr Rev. 2015.36(3):245-271.

- Are the GLP1 levels in Figure 2h and 5c active or total? The authors report a four-fold increase in plasma GLP1 concentration with the administration of their GIFT cells. GLP1 receptor agonists (RA) lead to a much higher increase in total compound GLP1 levels compared to endogenous GLP1 levels. However, the effect by the GIFT cells on metabolic control seems to recapitulate most of the effects observed with GLP1-RA. Can the authors provide an explanation for this discrepancy?

Reply:

Thank you for your valuable feedback. For context, in Figures 3e and 7c of the revised manuscript (previously Figure 2h and 5c), we now report the levels of active GLP-1 (*i.e.*, not total GLP-1, including both the active and inactive forms).

The seemingly comparable effects on metabolic control between GIFT cells and commercial GLP-1 RAs can be attributed to several factors. Firstly, the increase in plasma GLP-1 concentration observed with the administration of our GIFT cells specifically reflects the increase in biologically active GLP-1. At the same time, measurements involving GLP-1 RAs reported total GLP-1 levels, including both active and inactive peptide forms. Notably, GLP-1 naturally degrades rapidly in the bloodstream, and GLP-1RAs compensate for this by increasing the dosage to prolong their effect (Meier, *Nat Rev Endocrinol*, 2012). Consequently, injections of GLP-1 RAs often lead to significantly higher plasma GLP-1 levels than the levels necessary for their desired pharmacological effect. Our GIFT system is designed to provide a sustained release of active GLP-1 directly in response to high glucose levels, thereby avoiding the pulsatile dynamics often seen with injected GLP-1 RAs. Therefore, GIFT cells maintain GLP-1 at lower but therapeutically effective levels. Moreover, we speculate that the localized production of GLP-1 by GIFT cells in the gut likely enhances GLP-1 interaction with local GLP-1 receptors.

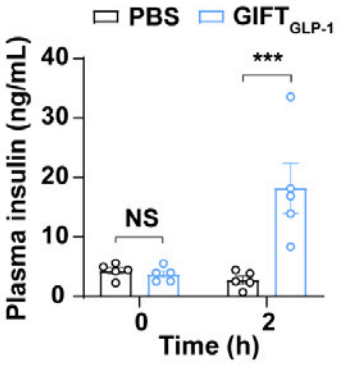
References:

Meier, J. GLP-1 receptor agonists for individualized treatment of type 2 diabetes mellitus. Nat Rev Endocrinol. 2012.8:728-742.

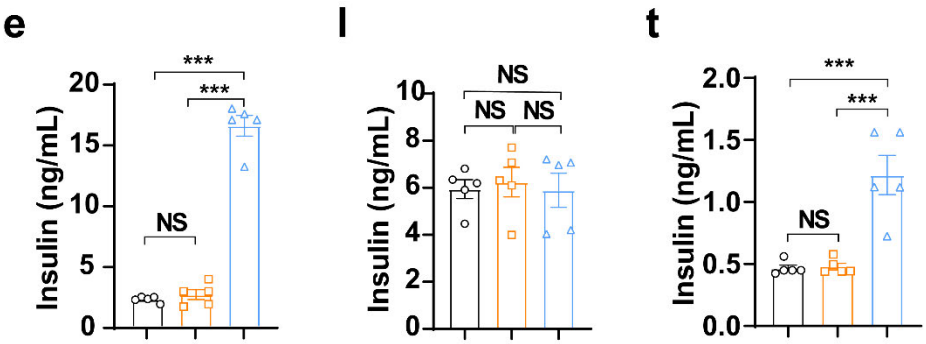
- In the acute setting (Fig. 2E-J, Figures 5), GLP-1 lowers plasma glucose via stimulation of glucose induced insulin secretion. Therefore, insulin values should be presented throughout the manuscript when GIFT is applied in vivo.

Reply:

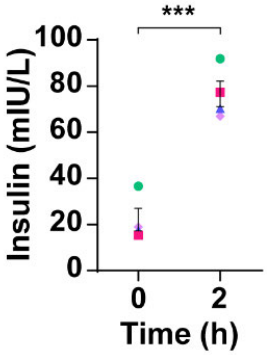
Following this guidance, we have included insulin measurements for all experiments in which GIFT was applied *in vivo*. Please see new **Fig. 3f**, **Fig. 6e**, **Fig. 6l**, **Fig. 6t**, and **Fig. 7d** in the revised manuscript and below:



Response Document Figure 28 (see Fig. 3f in the revised manuscript): GIFT$_{GLP-1}$ cells were administered to db/db mice via gavage at a dose of 10^9 CFU. Plasma insulin levels were measured at 2 hours after oral administration of GIFT$_{GLP-1}$ cells. Data are expressed as means ± SEM; $n = 5$ mice. P-values were calculated by two-way ANOVA. NS, not significant; ***$P < 0.001$.



Response Document Figure 29 (see Fig. 6e, l, t in the revised manuscript): e, Mice received daily oral gavage of PBS or GIFT$_{GLP-1}$ cells (10^9 CFU) for 30 days. As a control, db/db mice on a regular chow diet received a daily oral gavage of PBS for the same duration. Plasma insulin levels was measured 2 hours after the first gavage. **l,** DIO mice were treated with PBS, GIFT$_{Lux}$, or GIFT$_{GLP-1}$ cells (10^9 CFU) for 30 days. Plasma insulin levels was measured 2 hours after the first gavage. **t,** Diabetes was induced in high-fat diet-fed mice using streptozotocin. Mice were treated with PBS, GIFT$_{Lux}$, or GIFT$_{GLP-1}$ cells (10^9 CFU) for 30 days. Plasma insulin levels was measured 2 hours after the first gavage. Data are expressed as means ± SEM; $n = 5$ mice. P-values were calculated by two-way ANOVA. NS, not significant; ***$P < 0.001$.



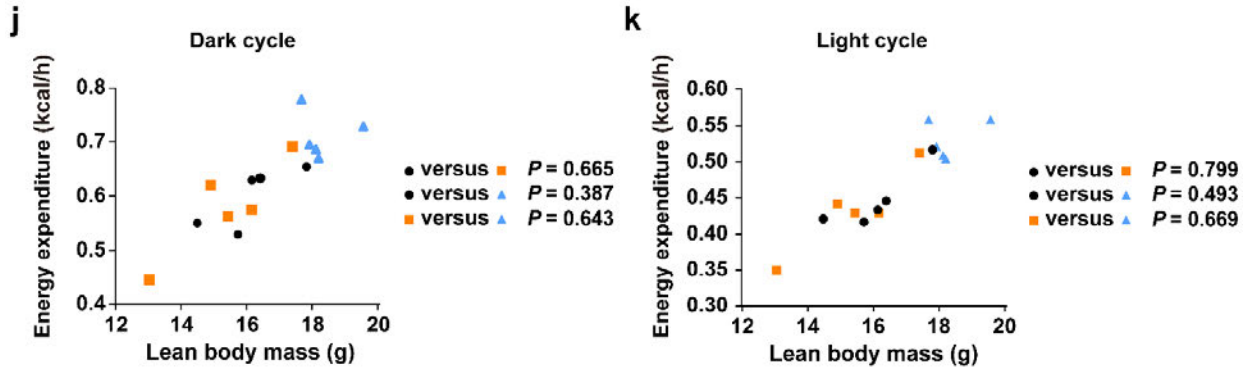
Response Document Figure 30 (see Fig. 7d in the revised manuscript): Four diabetic monkeys were orally administered GIFT$_{GLP-1}$ (2×10^{11} CFU/kg). Plasma insulin levels were measured 2 hours post-administration. Data are expressed as means ± SEM; $n = 4$ monkeys. P-values were calculated by t-test. ***$P < 0.001$.

- Figure 3 should include food intake data. The authors demonstrate an increase in energy expenditure that is not observed with GLP1 receptor agonists. An ANCOVA analysis should additionally be performed to analyze energy expenditure, which is the gold standard for analyzing this type of data in mice with different body weights. It would be helpful if the authors could provide an explanation for the discrepancy between their findings and the well-described effects of approved GLP1 receptor agonists on energy expenditure.

Reply:

We have relocated the food intake data from **Supplementary Fig. 9** to **Extended Data Fig. 7** (previously Figure 3) to ensure it is presented alongside the energy expenditure data for clarity and easy comparison. Compared to the control mice, db/db mice treated with $GIFT_{GLP\text{-}1}$ cells exhibited a decreased daily food intake. Additionally, we have conducted an ANCOVA analysis, incorporating lean body mass as a covariate to accurately analyze the energy expenditure data. Although db/db mice treated with $GIFT_{GLP\text{-}1}$ cells showed a significant increase in whole-body energy expenditure, when adjusted for lean body mass, their energy expenditure did not differ from control mice (**Extended Data Fig. 7j**, **k**). This finding suggests that the increase in energy expenditure observed in the $GIFT_{GLP\text{-}1}$ treated db/db mice is largely attributable to their increased lean body mass rather than an inherent enhancement of metabolic rate. Please see **page 11, lines 306-310** in the revised manuscript and below:

> "When normalized to lean mass, the energy expenditure of $GIFT_{GLP\text{-}1}$-treated mice was comparable to that of controls (**Extended Data Fig. 7j, k**). These findings collectively suggest that oral administration of $GIFT_{GLP\text{-}1}$ cells improves lipid profiles in db/db mice by suppressing food intake and enhancing whole-body lipid oxidation."



Response Document Figure 31 (see Extended Data Fig. 7 in the revised manuscript): Metabolic characterization of db/db mice treated with PBS, $GIFT_{Lux}$, or $GIFT_{GLP\text{-}1}$ for 30 days. Mice were individually housed in indirect calorimetry cages. **j**, **k**, Regression-based analysis of energy expenditure relative to lean body mass. *P*-values were calculated by ANCOVA using lean body mass as a covariate.

Regarding the discrepancy between our findings and the established effects of GLP-1 receptor agonists on whole-body energy expenditure, several factors may contribute to these differences. Firstly, the secretion of GLP-1 by our GIFT cells directly within the gut potentially influences local receptors and neuronal circuits differently than the systemic administration of GLP-1 receptor agonists, which

can lead to variations in the regulation of energy expenditure (McLean et al., *Endocr Rev*, 2021). Kindly note variations in responses to GLP-1-based therapies in the literature. For instance, intracerebroventricular injection of liraglutide has been shown to stimulate brown adipose tissue (BAT) thermogenesis and adipocyte browning in mice, independent of food intake (Beiroa et al., *Diabetes*, 2014). Similarly, intracerebroventricular co-administration of GLP-1 and GIP significantly increased neuronal activation and pro-opiomelanocortin (POMC) expression in the hypothalamic arcuate nucleus, suggesting enhanced metabolic activity (NamKoong et al., *Biochem Biophys Res Commun*, 2017). Moreover, in our study, the introduction of probiotics may modify the gut microbiota composition, which, when combined with the effects of GLP-1, could further influence energy expenditure.

References:

McLean B, Wong C, Campbell J, Hodson D, Trapp S, Drucker D. Revisiting the complexity of GLP-1 action-from sites of synthesis to receptor activation. Endocr Rev. 2021. 42(2):101-132.

Beiroa D, Imbernon M, Gallego R, Senra A, Herranz D, Villarroya F, Serrano M, Fernø J, Salvador J, Escalada J, Dieguez C, Lopez M, Frühbeck G, Nogueiras R. GLP-1 agonism stimulates brown adipose tissue thermogenesis and browning through hypothalamic AMPK. Diabetes. 2014. 63(10): 3346-58.

NamKoong C, Kim MS, Jang BT, Lee YH, Cho YM, Choi HJ. Central administration of GLP-1 and GIP decreases feeding in mice. Biochem Biophys Res Commun. 2017. 490(2): 247-252.

The authors state in the discussion that GIFT cells enhance insulin sensitivity in mice and NHPs. However, it is unclear whether this notion is solely based on the HOMA-IR index, which is used in humans and not validated for mice. Therefore, the authors should consider toning down this conclusion. Additionally, they should provide a more detailed explanation of what they mean by 'improved lipid metabolism'.

Reply:

Thank you for the insightful guidance here. As the HOMA-IR index has indeed not been validated for mice, we have removed it from the Results subsection about 'GIFT-mediated glycemic control in diabetic mice' of our manuscript, and describe the results as “alleviation of insulin resistance following GIFT cell treatment in mice and monkeys”. Please see **page 10, lines 264-266, page 18, lines 497-498,** and **page 19, lines 530-533** in the revised manuscript and below:

> “On day 31, we conducted glucose tolerance tests (GTT) and insulin tolerance tests (ITT) on db/db mice to assess the effects on glucose metabolism and insulin tolerance. A significant improvement in both glucose metabolism and insulin tolerance was observed in $GIFT_{GLP\text{-}1}$-treated db/db mice (**Fig. 3h-k**).”
>
> “Insulin resistance was also notably alleviated (**Fig. 7k**).”
>
> “Daily oral administration of the probiotics-based living drug in type 2 diabetic mice and monkeys provided increased GLP-1 secretion profiles compared to wild-type probiotic cells. This reduced blood glucose levels, improved postprandial glucose metabolism, and reduced insulin resistance.”

Regarding our intended meaning for 'improved lipid metabolism': first, please note that we have removed this phrase from the revised manuscript, and now present statements about four distinct lipid-related changes observed upon administration of $GIFT_{GLP-1}$: a decrease in overall fat mass, reductions in serum triglyceride and cholesterol levels, and a reduction in liver fat content. For context, we now understand that this phrase is excessively vague. Our use of this term was an unsuccessful attempt to amalgamate this diverse range of beneficial changes observed in our study subjects.

Please see **page 19, lines 533-535** in the revised manuscript and below:

> "Additionally, there were significant improvements in lipid profiles, including a decrease in overall fat mass, reductions in serum cholesterol and triglyceride levels, and reduced liver fat content."

Finally, the authors conclude that their approach provides "a feasible and facile administration route to promote long-term adherence". It would be helpful if they could describe the advantages over a once-weekly subcutaneous injection, considering that the GIFT cells only work temporarily and need to be replaced regularly. Additionally, it would be useful to know how this approach would work in humans.

Reply:

Thank you for focusing our attention here.

1. We appreciate the opportunity to clarify the advantages of GIFT cell therapy over once-weekly subcutaneous injections.

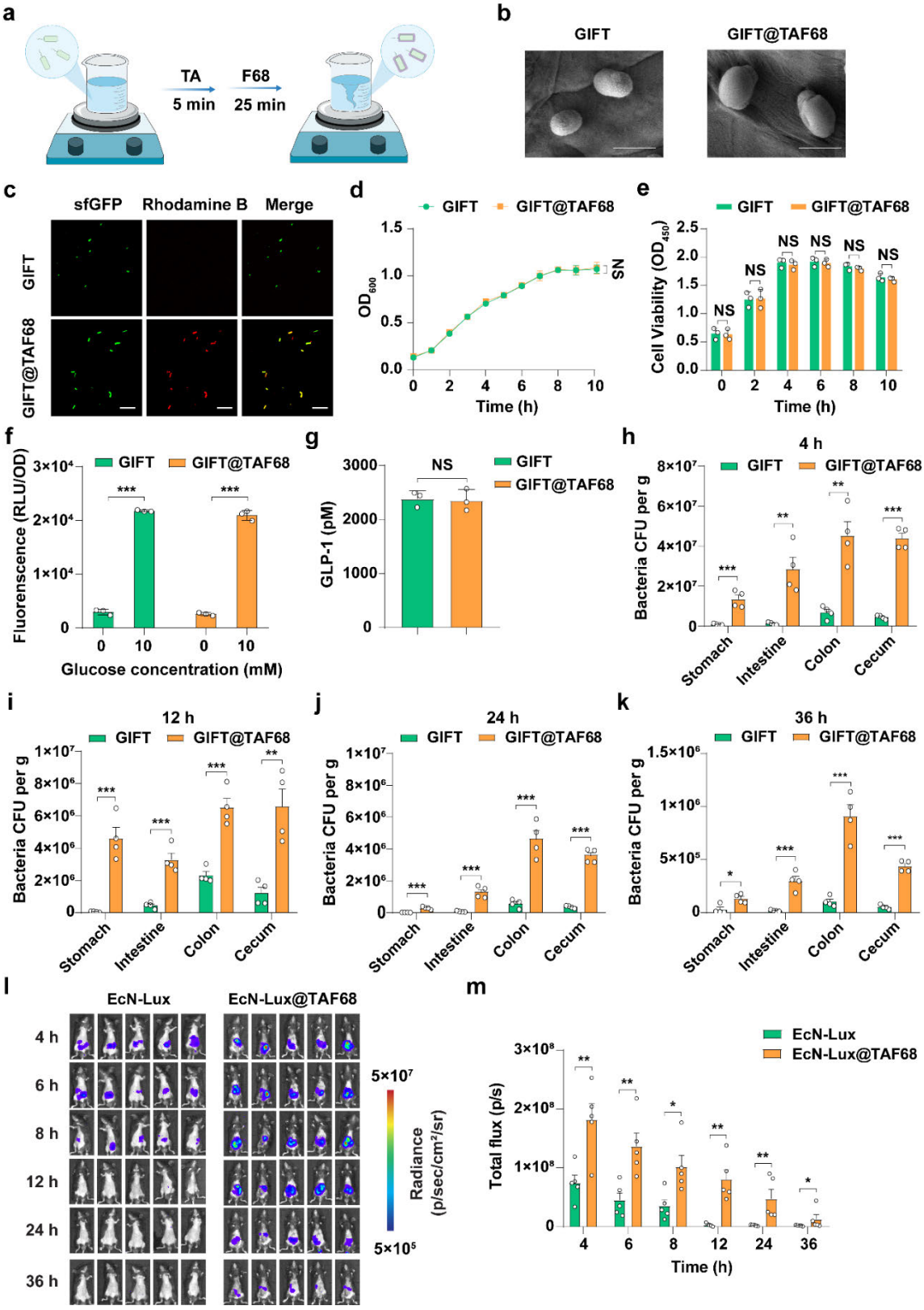
I. Reports in the literature frequently assert that an oral route of administration is more user-friendly than injections, which are the typical method for most GLP-1 receptor agonists currently in use. Indeed, a major reason for the high discontinuation rates of GLP-1 agonist therapies is the difficulty patients face with regular injections, underscoring the global effort towards developing oral GLP-1 therapies (Holman et al., *N Engl J Med*, 2017; Drucker, *Nat Rev Drug Discov*. 2020; Saxena et al., *Nat Med*, 2021). Moreover, we believe that engineered bacterial therapies, unlike long-term microbiota transplants, do not require ongoing customization. Further, we argue that the inability of EcN to colonize long-term should not be viewed solely as a drawback; instead, it can enhance safety and controllability (Kurtz et al., *Sci Transl Med*, 2019). Specifically, the transient nature of EcN colonization allows for temporal control over therapeutic interventions. Should any adverse effects arise, the effects of the cells can rapidly cease, thus allowing for immediate management of potential risks. Additionally, we consulted with clinical practitioners, who affirmed that a daily oral administration format is patient-friendly, easy to remember, and less prone to confusion in clinical practice.

We would also like to highlight that we have now completed new experiments exploring methods to extend colonization duration (which could, for example, minimize the dosage and frequency of administration). Specifically, we encapsulated the bacteria with tannic acid (TA) and poloxamer 188 (F68), which are FDA-approved pharmaceutical excipients (new **Extended Data Fig. 3a-c**). This

encapsulation significantly enhanced the intestinal colonization duration of the bacteria from 8 to 36 hours (new **Extended Data Fig. 3h-m**), without adversely affecting bacterial growth or metabolism (new **Extended Data Fig. 3d-g**). To further improve drug delivery efficiency, we developed a construct for expressing a tetra-copolymer of GLP-1 in GIFT cells. Briefly, the homo-monomers of the GLP-1 tetra-copolymer are linked via enterokinase cleavage sites. This design ensures that upon exposure to enterokinase, which is naturally present in the gut, the tetra-copolymer is efficiently cleaved into active GLP-1 monomers (new **Supplementary Fig. 9a**). *In vitro* experiments demonstrate that this configuration significantly enhances the production of active GLP-1 following cleavage by enterokinase (new **Supplementary Fig. 9b**). Please see **page 6, lines 170-176** and **page 13, lines 379-383** in the revised manuscript and below:

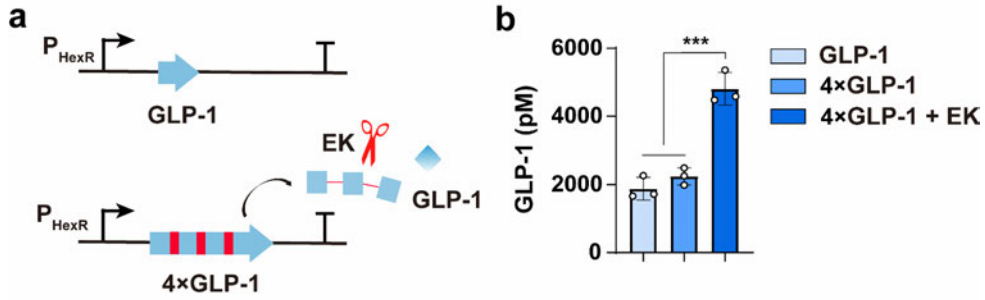
> "To prolong the residence time of GIFT cells in the gastrointestinal tract, we encapsulated the cells with tannic acid (TA) and poloxamer 188 (F68), both FDA-approved excipients[43] (**Extended Data Fig. 3a-c**). This encapsulation did not negatively impact bacterial growth or metabolism (**Extended Data Fig. 3d, e**), nor did it affect system dynamics or therapeutic protein secretion (**Extended Data Fig. 3f, g**). Additionally, encapsulation with TA and F68 extended the intestinal colonization of GIFT cells in mice from 8 to 36 hours, as shown by *in vivo* imaging (**Extended Data Fig. 3h-m**)."

> "To improve drug delivery, we engineered $GIFT_{GLP\text{-}1}$ cells to express a GLP-1 tetra-copolymer, with its monomers linked by enterokinase cleavage sites, allowing activation by enterokinase, an enzyme naturally present in the gut (**Supplementary Fig. 9a**). *In vitro* tests showed that this design significantly increased active GLP-1 production following cleavage by enterokinase (**Supplementary Fig. 9b**)."



Response Document Figure 32 (see Extended Data Fig. 3 in the revised manuscript): Characterization of GIFT intestinal colonization coated with tannic acid (TA) and poloxamer 188 (F68). a, Schematic representation of the preparation process for coated GIFT (GIFT@TAF68). $GIFT_{GFP}$ cells were sequentially treated with TA and F68 for 5 min, followed by an additional 25 min of F68 treatment with continuous stirring throughout the encapsulation process. **b**, Scanning electron microscope (SEM) images of uncoated $GIFT_{GFP}$ and

$GIFT_{GFP}$@TAF68. Scale bar, 1 μm. **c**, Confocal scanning laser microscopy (CSLM) images of $GIFT_{GFP}$ and $GIFT_{GFP}$@TAF68. The red channel represents rhodamine B-labeled TA, while the green channel shows sfGFP expressed by $GIFT_{GFP}$. Scale bar, 10 μm. **d**, Growth curves of $GIFT_{GFP}$ and $GIFT_{GFP}$@TAF68 cultured in LB broth at 37 °C. OD_{600} was recorded using a microplate reader. **e**, Cell viability of $GIFT_{GFP}$ and $GIFT_{GFP}$@TAF68. **f**, Quantification of sfGFP expression by $GIFT_{GFP}$ and $GIFT_{GFP}$@TAF68 after 12 hours of culture with 10 mM glucose. **g**, Measurement of GLP-1 secretion by $GIFT_{GLP\text{-}1}$ and $GIFT_{GLP\text{-}1}$@TAF68 after 12 hours of culture with 15 mM glucose. **h-k**, Bacterial colony counts in the gastrointestinal tract of mice. C57BL/6 mice were orally administrated 10^9 CFU of either $GIFT_{GFP}$ or $GIFT_{GFP}$@TAF68 cells. Tissue samples from the stomach, small intestine, colon, and cecum were harvested at 4 (**h**), 12 (**i**), 24 (**j**), and 36 hours (**k**) post-administration, samples were homogenized and plated on LB agar. After 12 hours of incubation at 37 °C, bacterial colonies were counted. **l**, Bioluminescence images of mice. EcN constitutively expressing LuxCDABE (EcN-Lux) (10^9 CFU) were orally administrated to C57BL/6 mice, and imaging was performed at 4, 6, 8, 12, 24, and 36 hours using an *in vivo* imaging system. **m**, Quantification of bioluminescence signals from **l**. Data in **d-g** are expressed as means ± SD; $n = 3$ independent experiments. Data in **h-k** and **m** are expressed as means ± SEM; $n = 4$ mice for **h-k**, $n = 5$ mice for **m**. *P*-values were calculated using a two-tailed unpaired *t*-test. NS, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.



Response Document Figure 33 (see Supplementary Fig. 9a, b in the revised manuscript): Design of a construct for GLP-1 tetra-copolymer expression in GIFT cells. a, Schematic of the genetic configurations for GLP-1 and tetrameric GLP-1 (4×GLP-1) expression systems. The 4×GLP-1 is linked via enterokinase (EK) cleavage sites. **b**, Quantification of GLP-1 levels in the culture supernatant from $GIFT_{GLP\text{-}1}$ cells expressing either GLP-1 or 4×GLP-1. Cells (10^9 CFU) were induced with 15 mM glucose for 12 hours, followed by incubation of the supernatant with enterokinase for 12 hours to activate tetrameric GLP-1. GLP-1 content was then measured using an ELISA kit**.** Data in **b** are expressed as means ± SD; $n = 3$ independent experiments. *P*-values were calculated using one-way ANOVA. $***P < 0.001$.

II. Numerous studies and clinical trials have highlighted a range of adverse effects associated with the systemic administration of GLP-1 receptor agonists, such as gastrointestinal discomfort, pancreatitis, pancreatic cancer, and thyroid complications, among others (Sodhi et al., *JAMA*, 2023; Gier et al., *Diabetes*, 2012; Gier, *Gastroenterology*. 2011; Gier et al., *J Clin Endocrinol Metab*, 2012). To extend the half-life of GLP-1 receptor agonists, these agents are often modified with lipid-like structures. While these modifications successfully improve drug efficacy, they may also lead to the development of resistance or allergic reactions (Liu et al., *J Pharmacol Toxicol Methods*, 2011; Garay et al., *Expert Opin Drug Deliv*, 2012; Schellekens, *Pharm Res*. 2013). Our engineered probiotic system is

specifically designed to mitigate these risks by providing controlled expression of GLP-1, potentially reducing overall exposure compared to systemic delivery. To evaluate the potential adverse effects associated with systemic semaglutide (a clinically approved GLP-1 receptor agonist used for the treatment of type 2 diabetes and obesity, administered as a once-weekly injectable) injections versus the targeted, controlled delivery of GLP-1 via GIFT, we conducted a series of evaluations in db/db mice.

First, to assess acute inflammatory and hypersensitivity reactions, blood samples of db/db mice receiving a single administration of semaglutide or $GIFT_{GLP\text{-}1}$ were collected for complete blood count, serum biochemical analysis, and analysis of immunoglobulin E (IgE) and calcitonin levels (new **Extended Data Fig. 10a** and **Supplementary Tables 1**, **2**). Semaglutide (1 mg/kg) injection led to a host of physiological impacts, none of which were observed for the mice given oral GIFT administration. Specifically, the semaglutide group mice had significantly elevated specific IgE levels at 6 hours after dosing, indicative of an immediate hypersensitivity reaction (new **Extended Data Fig. 10b**). Increases in plasma calcitonin levels were observed at 12 and 24 hours after a single dose of semaglutide, suggesting potential adverse effects on the thyroid gland (new **Extended Data Fig. 10c**, **d**). Additionally, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly elevated at 12 hours after semaglutide injection but returned to normal levels by 24 hours, indicating an acute hepatic stress response (new **Extended Data Fig. 10e-h**). Moreover, serum IL-6 and IFN-β levels were significantly increased at 6 hours after injection, indicating an acute inflammatory response (new **Extended Data Fig. 10i**, **j**).

We also performed a conditioned taste aversion (CTA) study in db/db mice to investigate the adverse responses elicited by $GIFT_{GLP\text{-}1}$ and semaglutide (new **Extended Data Fig. 10k**). CTA is a highly specialized form of associative learning in which animals learn to avoid a taste that is associated with a detrimental health condition, demonstrating its utility for assessing potential aversive effects of treatments in a medical context (Garcia et al., *Science*, 1955). The mice were exposed to a highly palatable 1% sucrose solution, followed by administration of $GIFT_{GLP\text{-}1}$ (10^9 CFU) or semaglutide (0.12 mg/kg or 1 mg/kg). After the administration, the mice were then exposed to a two-bottle taste preference test, offering a voluntary choice between tap water and a 1% sucrose solution. The consumption of sucrose and water was monitored for 24 hours. Compared to $GIFT_{GLP\text{-}1}$, we found that semaglutide induced a significantly greater aversive response to the sucrose solution (new **Extended Data Fig. 10l-n**).

Additionally, we compared the risk of hypoglycemia when both treatments were used in combination with insulin glargine. High doses of semaglutide (1 mg/kg, based on human dosage conversion) and low doses of semaglutide (0.12 mg/kg, referenced from literature) combined with insulin glargine (7 U/kg) each resulted in significant increases in the incidence of hypoglycemia; no increased hypoglycemic events were observed when GIFT was used in combination with insulin glargine (7 U/kg) (new **Supplementary Tables 3-9**).

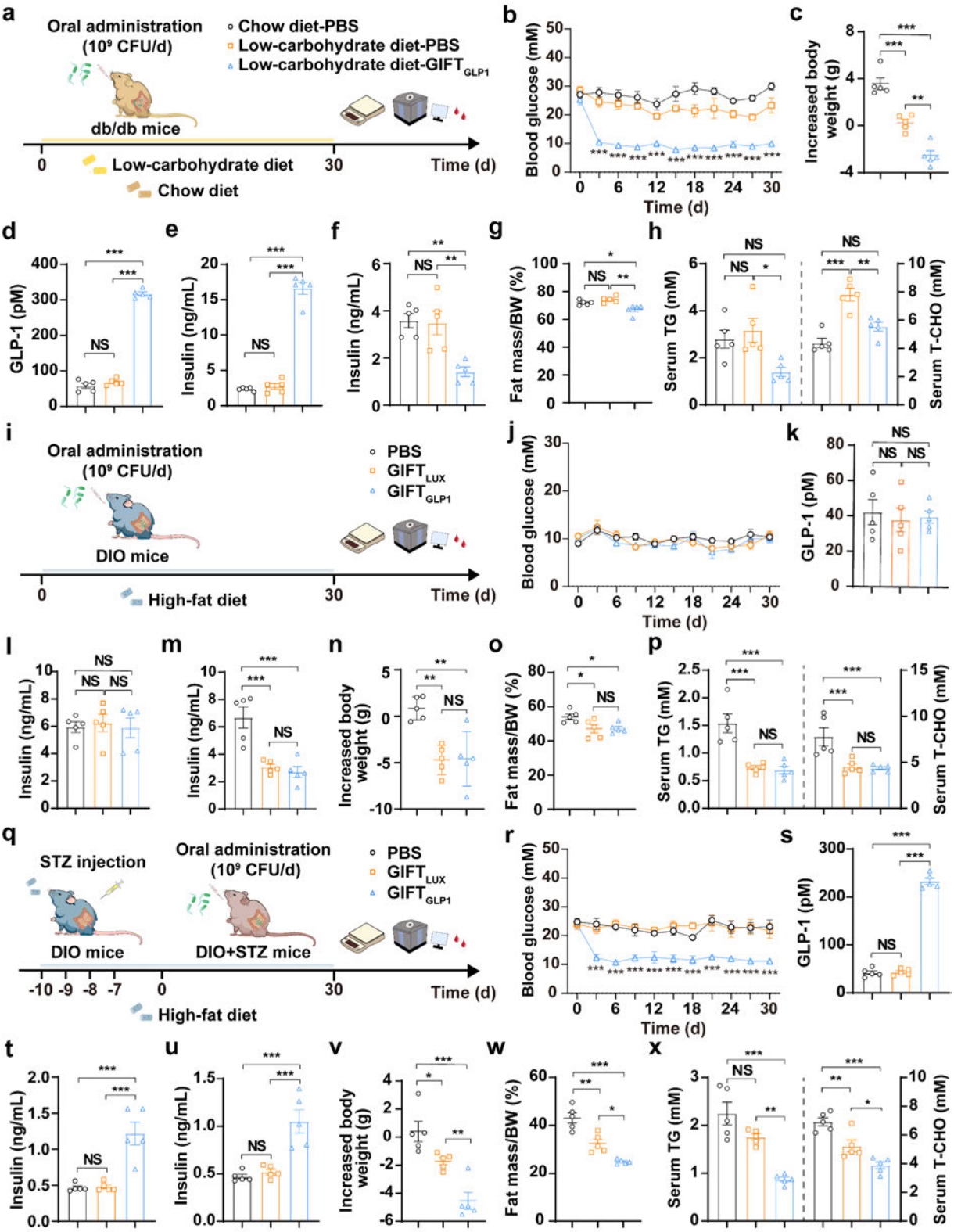
These findings support the advantages of the GIFT system over a once-weekly injectable intervention in reducing systemic side effects while effectively managing glucose levels. Please see **pages 16-17, lines 446-483** in the revised manuscript and below:

"We assessed acute inflammatory and hypersensitivity reactions in db/db mice following a single administration of $GIFT_{GLP\text{-}1}$ (10^9 CFU) or semaglutide (1 mg/kg). Blood samples were

collected for complete blood count, serum biochemistry, and analysis of immunoglobulin E (IgE), calcitonin, interleukin-6 (IL-6), and interferon beta (IFN-β) levels (**Extended Data Fig. 10a** and **Supplementary Tables 1, 2**). Semaglutide injections triggered several physiological responses not observed in the GIFT group. Specifically, semaglutide-treated mice exhibited significantly elevated IgE levels at 6 hours after dosing, indicating an immediate hypersensitivity reaction (**Extended Data Fig. 10b**). Plasma calcitonin levels also increased at 12 and 24 hours, suggesting potential adverse effects on the thyroid gland (**Extended Data Fig. 10c**, **d**). Elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels at 12 hours post-injection indicated acute hepatic stress, which resolved by 24 hours (**Extended Data Fig. 10e-h**). Serum IL-6 and IFN-β levels were significantly increased at 6 hours after injection, indicating an acute inflammatory response (**Extended Data Fig. 10i**, **j**).

To assess potential adverse responses elicited by $GIFT_{GLP\text{-}1}$ and semaglutide, we conducted a conditioned-taste aversion (CTA) study in db/db mice. CTA is a specialized form of associative learning where animals avoid a taste linked to a detrimental health condition[64]. Mice were given a 1% sucrose solution, followed by administration of $GIFT_{GLP\text{-}1}$ (10^9 CFU) or semaglutide (0.12 mg/kg or 1 mg/kg) (**Extended Data Fig. 10k**). A two-bottle taste preference test was then performed, offering mice a choice between tap water and the sucrose solution, with consumption monitored over 24 hours. Despite semaglutide-treated mice displaying similar glycemic control levels compared to those treated with $GIFT_{GLP\text{-}1}$ 4 hours after administration (**Extended Data Fig. 10l**), they demonstrated a significantly stronger aversive response to the sucrose solution (**Extended Data Fig. 10m**). Moreover, semaglutide-treated mice consumed significantly less water compared to both untreated mice and those treated with $GIFT_{GLP\text{-}1}$ cells (**Extended Data Fig. 10n**), indicating potential adverse side effects of semaglutide.

Given the complexity of diabetes management, often requiring combination therapies, we assessed the risk of hypoglycemia when $GIFT_{GLP\text{-}1}$ or semaglutide were combined with insulin glargine. db/db mice received insulin glargine alone (7 U/kg), high-dose semaglutide (1 mg/kg, adjusted from human dosages), low-dose semaglutide (0.12 mg/kg, as reported in literature), or $GIFT_{GLP\text{-}1}$ (10^9 CFU). Insulin glargine alone resulted in an approximately 20% risk of hypoglycemia (**Supplementary Table 3**). When combined with insulin glargine, both high and low doses of semaglutide significantly increased hypoglycemia events (**Supplementary Tables 4-7**). In contrast, no increase in hypoglycemia was observed with the combination of $GIFT_{GLP\text{-}1}$ and insulin glargine (**Supplementary Tables 8**, **9**), highlighting the advantage of glucose-responsive drug release. Thus, GIFT cells demonstrate a superior safety profile and fewer behavioral side effects compared to semaglutide in mice."



Response Document Figure 34 (see Extended Data Fig. 10 in the revised manuscript): Comparative analysis of adverse effects between semaglutide injection and oral delivery of GIFT$_{GLP-1}$ in db/db mice. a, Schematic of the timeline for assessing acute adverse effects. db/db mice were divided into three groups: one received oral GIFT$_{GLP-1}$, another was injected with semaglutide, and the third group remained untreated. Blood samples were collected at 6 hours for serum immunoglobulin E (IgE), interleukin-6 (IL-6), and Interferon beta (IFN-β) analysis, and at 12 and 24 hours for complete blood count (CBC), serum biochemical analysis, and

calcitonin (CT) measurement. **b**, Serum IgE levels in db/db mice 6 hours after treatment. **c-h**, Serum levels of CT (**c**, **d**), alanine transaminase (ALT) (**e**, **f**), and aspartate transaminase (AST) (**g**, **h**) in db/db mice 12 and 24 hours after treatment. **i**, **j**, Serum levels of IL-6 (**i**) and IFN-β (**j**) in db/db mice 6 hours after treatment. **k**, Schematic of the timeline for the conditioned taste aversion (CTA) experiment. db/db mice were single-housed with *ad libitum* access to chow diet and tap water for 48 hours, followed by access to 1% sucrose for 24 hours. After acclimation, mice given oral $GIFT_{GLP\text{-}1}$ or injected with semaglutide underwent a two-bottle preference test, choosing between tap water and 1% sucrose. Sucrose and water intake were recorded for 24 hours. **l**, Blood glucose levels monitored 4 hours after $GIFT_{GLP\text{-}1}$ or semaglutide administration. **m**, Total sucrose intake. **n**, Total water intake. Data in **b-j** and **l-n** are expressed as means ± SEM; $n = 6$ mice in **b-j**, $n = 9$ mice in **l-n**. *P*-values were calculated using one-way ANOVA. NS, not significant; $*P < 0.05$, $**P < 0.01$, $**P < 0.001$.

2. Thank you for your interest in how this approach could work in humans.

Based on extensive animal studies, we hypothesize that GIFT cells hold potential for efficacy in various patient populations. For example, in type 2 diabetes patients, oral administration of GIFT cells could help maintain glucose homeostasis, as suggested by our results in a non-genetic type 2 diabetes mouse model, which is highly representative of human type 2 diabetes (new **Fig. 6q-x**). Additionally, in diet induced obesity, our GIFT cells have shown potential for weight reduction (new **Fig. 6i-p**). For healthy individuals with a high-sugar diet, GIFT could also serve as a functional supplement to mitigate blood glucose fluctuations (new **Fig. 5g-k**).

While our study has shown promising results in mice and non-human primates, transitioning to the evaluation of GIFT cells in human subjects will necessitate extensive preparatory work, including, for example, collection of preclinical data, including detailed pharmacokinetic and pharmacodynamic studies and toxicity assessments at varying dosages across multiple animal models, to meet clinical trial application requirements. Specifically, we need to monitor serum levels of GLP-1 and insulin at various times following administration of low, medium, and high dosages of GIFT cells in diabetic model animals, particularly in NHPs, which provide the closest approximation of human responses. Moreover, assessing the colonization and tolerability of GIFT cells in NHPs is essential. Provided that it makes good sense to proceed after completing such preclinical evaluations, we would design early-phase clinical trials targeting a small group of diabetic patients. Initially, we aim to enhance the stability of our live biotherapeutic products through bacterial chassis engineering and optimization of production processes, followed by small-scale production under Good Manufacturing Practice (GMP) guidelines. Given that diet significantly influences gut microbiota and the host's metabolic profile, integrating dietary guidelines into the treatment regimen will likely be essential. Through continuous monitoring of the activity and persistence of GIFT cells in the human gut, alongside evaluations of potential immune responses, changes in gut microbiota composition, and overall health impacts, we would be positioned to understand the long-term effects of chronic GIFT cell administration.

References:

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Minor:

Abstract: The term "hypoglycemic" agents is not correct; it should be glucose lowering agents.

Reply:

Thank you for bringing this error to our attention. We have replaced the incorrect "hypoglycemic agents" with "glucose-lowering agents" in the abstract. Please see **page 1, line 22** in the revised manuscript and below:

"Sustained and controlled delivery of glucose-lowering agents using engineered designer cells is recognized as an effective strategy for diabetes therapy."

Introduction: "antidiabetic drugs" should be replaced by "glucose lowering drugs".

Reply:

We have replaced the term "antidiabetic drugs" with "glucose-lowering drugs" in the introduction. Please see **page 3, line 81** in the revised manuscript and below:

"However, to date, no research has been conducted on oral probiotic-based medications capable of delivering glucose-lowering drugs in response to glucose sensing."

Again, we express our sincere gratitude for the helpful guidance you provided in improving our study and manuscript. Many thanks!

Nature 2023-10-18581A: Point-by-point response

We would like to thank the Editor and both Referees for their thoughtful comments and constructive guidance, which have significantly helped us improve the quality of our study. We provide point-by-point responses to each of the referee comments (below). We start by summarizing the major updates from our revision process.

1. We constructed a strong constitutive GLP-1 expression strain (EcN-P_{tac}-GLP-1) and compared its GLP-1 secretion profile, genetic stability, and therapeutic efficacy against $GIFT_{GLP\text{-}1}$. The EcN-P_{tac}-GLP-1 strain had inclusion bodies, and showed reduced GLP-1 secretion (bioactivity) and genetic instability (**new Supplementary Fig. 6, 7**). These findings support the notion that sustained GLP-1 overexpression carries a substantial metabolic burden and is not suitable for therapeutical application.

2. We tested two moderate-strength constitutive promoters (P_{lacUV5} and P_{lac}), which resulted more stable expression but insufficient GLP-1 levels to achieve therapeutic glucose lowering (**new Supplementary Fig. 7a-c**). Viewed alongside our $GIFT_{GLP\text{-}1}$ results, these findings support the superior efficacy of using a glucose-responsive system, in terms of both GLP-1 secretion and glycemic control.

3. We measured lean mass using MRI-based body composition analysis. While semaglutide-treated mice showed a trend toward lean mass reduction, the $GIFT_{GLP\text{-}1}$-treated mice showed well maintained or modestly increased lean mass (**new Extended Data Fig. 10o**), suggesting a potential advantage of $GIFT_{GLP\text{-}1}$ in preserving lean tissue during periods of fat mass reduction.

4. We conducted a two-month comparative safety study of $GIFT_{GLP\text{-}1}$ and semaglutide treatment in db/db mice. Complete blood counts (**new Supplementary Table 10**), serum biochemistry (**new Supplementary Table 11**), and proinflammatory cytokine levels (**new Extended Data Fig. 10p-s**) revealed no significant differences between the two groups, supporting the favorable long-term safety and tolerability of our orally delivered therapeutic system.

5. We have added new data comparing glycemia levels among healthy monkeys, untreated diabetic monkeys, and $GIFT_{GLP\text{-}1}$-treated diabetic monkeys. Briefly, oral administration of $GIFT_{GLP\text{-}1}$ effectively and sustainably maintained blood glucose at a relatively low level (**new Fig. 7h**), findings providing clear evidence of therapeutic benefit.

6. We inhibited the Sec secretion pathway in $GIFT_{GLP\text{-}1}$ cells using a SecA inhibitor, which resulted in a significant reduction in GLP-1 levels in the culture supernatant (**new Supplementary Fig. 5a-c**). These results support that GLP-1 secretion is dependent on both the LamB signal peptide and the Sec pathway.

7. We quantified GLP-1 secretion efficiency in $GIFT_{GLP\text{-}1}$ cells using ELISA. And we found that approximately 33.3% of the total GLP-1 was released into the culture supernatant (**new Supplementary Fig. 5d**).

8. We constructed a control strain expressing GLP-1 without a secretion signal peptide to evaluate the contribution of bacterial lysis to GLP-1 release. This strain exhibited minimal extracellular GLP-1, and oral administration failed to reduce blood glucose levels in db/db mice (**new Supplementary Fig. 5e, h**), indicating that lysis-derived GLP-1 did not contribute to the beneficial effects in our $GIFT_{GLP-1}$.

9. We measured the biological activity of lysis-derived GLP-1 and found it to be reduced, likely due to the retention of the uncleaved signal peptide, which prevents the generation of the mature GLP-1 fragment (residues 7-37) (**new Supplementary Fig. 5f, g**). These findings collectively demonstrate that the therapeutic effect of $GIFT_{GLP-1}$ is driven by the secretion of mature, bioactive GLP-1 mediated by the LamB signal peptide, rather than by release through bacterial lysis.

10. We administered one or two oral glucose gavages to wild-type mice harboring $GIFT_{GLP-1}$ cells and monitored serum GLP-1 levels over time. A single gavage induced a transient GLP-1 increase, while two consecutive gavages led to sustained elevation (**new Supplementary Fig. 9**). These results demonstrate that $GIFT_{GLP-1}$ cells dynamically respond to glucose fluctuations *in vivo*.

Referee #1 (Remarks to the Author):

* The HexR system was also characterized in *P. putida* (PMID: 34508142) and I do not believe this in *E. coli* is a significant advance as the authors have described it.

Reply:

Thank you for your comment. We acknowledge that a glucose-inducible HexR system has previously been characterized in *Pseudomonas putida* (*Sci Rep* 2021, PMID: 34508142). However, the previous reported system exhibited a very narrow glucose response, with no significant changes in promoter activity beyond 5 mM glucose. Thus, the previous reported system did not have any potential for therapeutical application for glycemia control (PMID: 32310436).

Based on the native glucose-inducible HexR system, we have substantially improved and re-engineered a completely novel glucose-responsive gene regulation system through (1) codon optimization and functional tuning of HexR to enhance compatibility and responsiveness in *E. coli*, (2) screening and refinement of HexR-binding DNA motifs to improve transcriptional regulation, and (3) promoter engineering to construct synthetic promoters responsive across a physiologically relevant glucose range. These efforts integrated genetic elements from both *P. putida* and *E. coli*, resulting in a synthetic system with fundamentally distinct regulatory characteristics compared to the native HexR system. Specifically, gene transcription was minimally activated at glucose concentrations below 5 mM, increased with rising glucose levels, and reached a 48.8-fold induction relative to background at 20 mM glucose.

The innovation of our study lies not in identifying glucose-responsive elements *per se*, but in repurposing and optimizing the HexR system to construct **a living, self-regulating therapeutic platform** capable of dynamically adjusting therapeutic protein production.

This work represents a conceptual and technological advance in the field of live biotherapeutics. Recent recognition of “glucose-sensitive insulin” as a top scientific breakthrough (*Nature* 2024, PMID: 39415004; Nature’s list of 2024) highlights the growing emphasis on precision, feedback-controlled treatments that respond to physiological signals. In this context, our work introduces a living therapeutic system that autonomously senses and responds to hyperglycemia. This approach fundamentally differs from conventional drug delivery systems in three ways:

—**Real-time, self-regulating glycemic control:** Unlike passive drug administration, our system continuously monitors glucose fluctuations and dynamically adjusts GLP-1 production in a controlled, feedback-regulated manner.

—**Reduced risk of adverse effects:** Precise, glucose-dependent GLP-1 production helps minimize side effects such as hypoglycemia (**Supplementary Tables 3–9**), acute inflammatory responses, and hypersensitivity reactions associated with excessive or unregulated GLP-1 production (**Extended Data Fig. 10; Supplementary Tables 1,2**).

—**A compliance-friendly therapeutic solution:** The "living drug" nature of our system enables on-demand biopharmaceutical production within the gut, presenting a promising alternative to invasive treatments (*e.g.*, GLP-1 receptor agonist injections) for chronic conditions like diabetes.

Thus, while HexR-based regulation in *P. putida* has been previously described, our demonstration of a **living, self-regulating therapeutic platform** through GLP-1 modulation serves as a proof-of-concept for cell-based therapies that respond precisely to internal metabolites. Looking ahead, the modular gene regulatory architecture we established-comprising synthetic, metabolite-responsive promoters and transcription factors-provides a framework for engineering designer probiotics that sense and respond to specific host-derived metabolites. With the incorporation of appropriate regulatory sensors (*e.g.*, transcriptional repressors or activators responsive to uric acid or cholesterol), this strategy could be extended to the treatment of chronic diseases like gout, hyperlipidemia, and other metabolic, inflammatory, and endocrine disorders.

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Hoeg-Jensen T, Kruse T, Brand CL, Sturis J, Fledelius C, Nielsen PK, Nishimura E, et al. Glucose-sensitive insulin with attenuation of hypoglycaemia. ***Nature***. 2024.634(8035):944-951. PMID: 39415004.

* I did not see a proper control for a constitutive GLP production system and its effect on mice. Since the bacteria release system is different from the normal route of administration of GLP, additional characterization of the toxicity needs to be demonstrated. I don't believe the authors have clearly demonstrated a need for a glucose-sensitive system.

Reply:

I. Regarding the control for a constitutive GLP production system:

Thank you for your feedback. In this revised version, we have included a system with constitutive GLP-1 production as a control for our $GIFT_{GLP-1}$. The two systems differ in that GLP-1 production is glucose-dependent in $GIFT_{GLP-1}$, whereas it is glucose-independent in the control system. After detailed comparing the control and our $GIFT_{GLP-1}$ system, we concluded that the glucose-sensitive system is definitely required for a better efficacy, efficiency and genetic stability.

We investigated the efficacy and feasibility of constitutive GLP-1 expression systems as controls. We initially constructed engineered EcN cells transformed with a strong constitutive GLP-1 expression system (P_{tac}-GLP-1) and evaluated GLP-1 secretion as well as blood glucose levels in db/db mice compared to those of our glucose-responsive GLP-1 secretion cells ($GIFT_{GLP-1}$). While GLP-1 levels and glucose-lowering effects were comparable between the two groups (**new Supplementary Fig. 7a-c**), transmission electron microscopy revealed the formation of inclusion bodies in cells harboring the strong constitutive construct (**new Supplementary Fig. 6**), indicating substantial metabolic burden on the host bacteria when sustained secretion of exogenous functional proteins (*Microb Cell Fact* 2024,

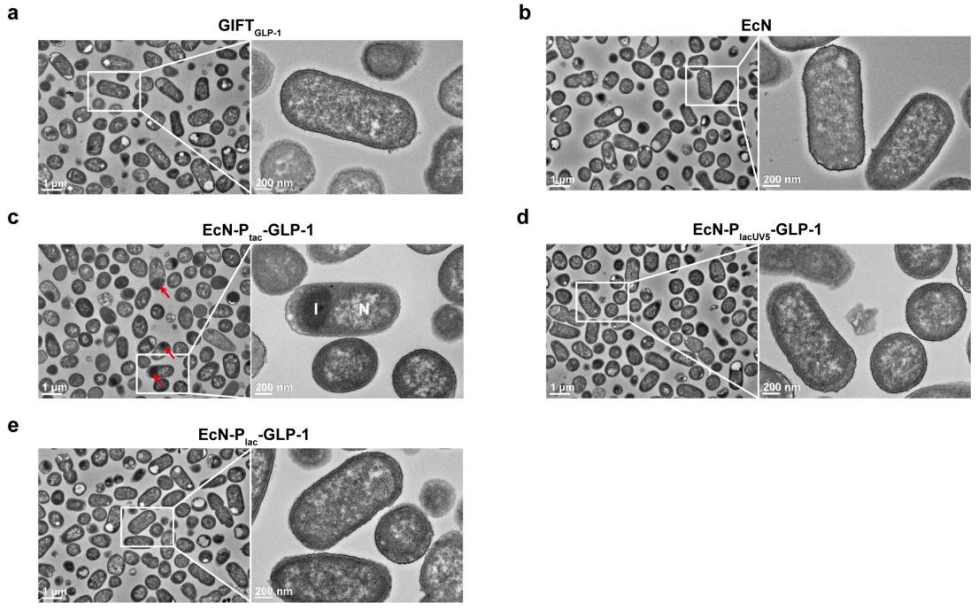
PMID: 38555441; *Nat Rev Microbiol* 2004, PMID: 15035009).

We also observed a progressive decline in both GLP-1 levels and bioactivity in the supernatant from constitutive strains across successive *in vitro* passages, accompanied by a reduced glucose-lowering effect in mice (**new Supplementary Fig. 7d-i**). In contrast, no such decline occurred with the glucose-responsive system. Plasmid sequencing of the 9th-passage constitutive GLP-1 expression strain revealed multiple mutations (**Response Document Fig. 1**), demonstrating genetic instability under continuous high-level expression. We interpret these findings as aligning with a (to our understanding) broader consensus that inducible systems are more effective than constitutive systems for the secretion of exogenous functional proteins, as they can better manage protein expression levels and reduce the metabolic burden on host cells (*Microb Cell Fact* 2024, PMID: 38555441; *Nat Rev Microbiol* 2004, PMID: 15035009).

To provide a meaningful benchmark, we also tested moderate-strength constitutive promoters (P_{lacUV5} and P_{lac}). These constructs yielded more stable expression; however, GLP-1 secretion levels were insufficient to achieve therapeutic glucose-lowering effects in db/db mice (**new Supplementary Fig. 7a-c**).

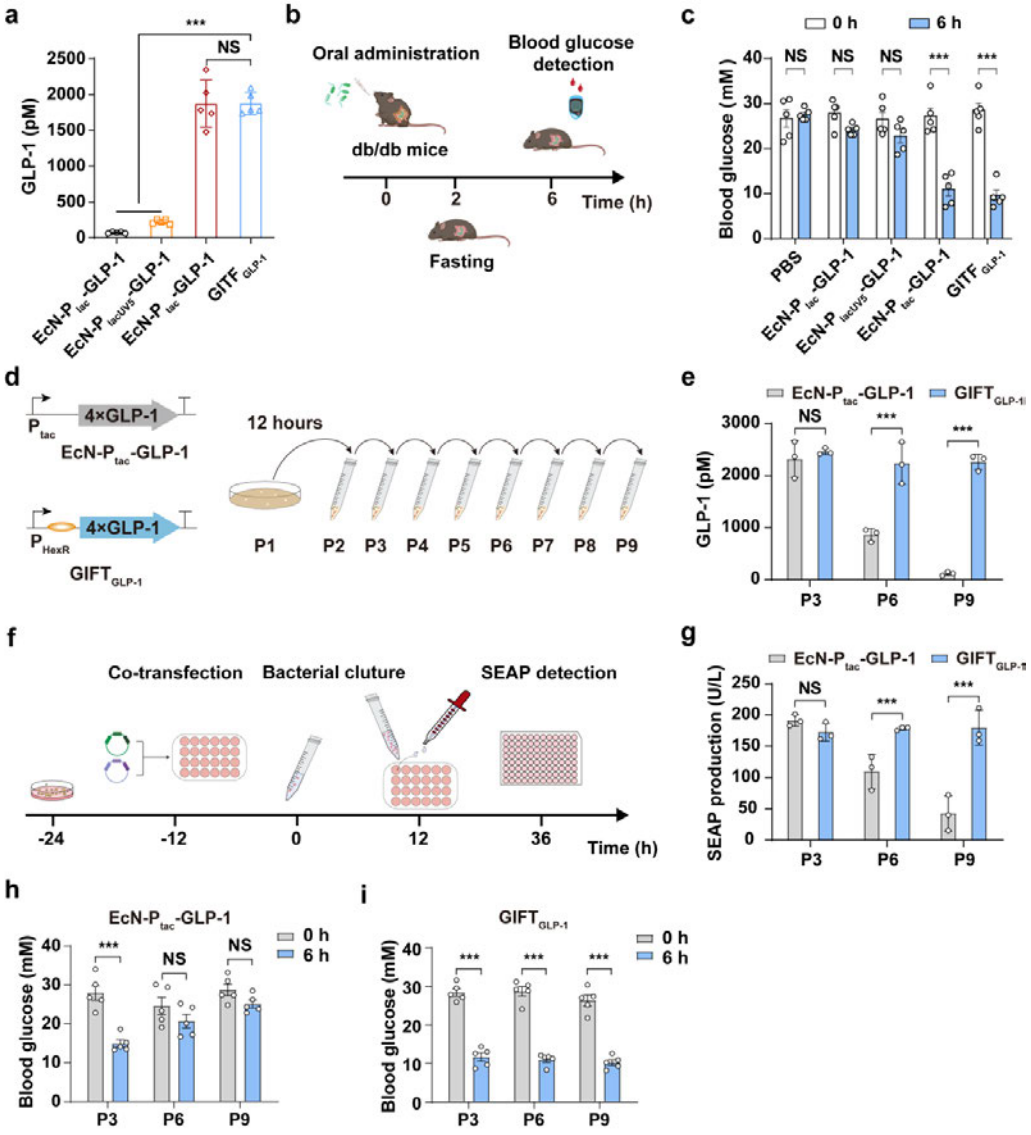
Collectively, the glucose-responsive system demonstrates superior efficacy for GLP-1 secretion and blood glucose lowering in mice over both high- and moderate-strength constitutive expression systems. These findings have been incorporated into our revised manuscript. Please see **page 9, lines 259-262** in the revised manuscript and below:

> "Glucose-responsive regulation of GLP-1 expression confers greater stability compared to constitutive GLP-1 expression systems, resulting in enhanced GLP-1 secretion and more sustained glycemic control in db/db mice (**Supplementary Fig. 6a-e, 7a-i**)."



Supplementary Fig. 6: Transmission electron microscopy (TEM) images showing inclusion body formation in engineered EcN cells. $GIFT_{GLP-1}$ (**a**), EcN (**b**), and EcN transformed with constitutive GLP-1 expression plasmids driven by P_{tac} (**c**), P_{lacUV5} (**d**), or P_{lac}

(e) were cultured for 12 h before TEM analysis. Red arrows indicate the location of inclusion bodies. Inclusion bodies (I) and the nuclear region (N) are indicated.



Supplementary Fig. 7. A comparison between inducible and constitutive expression of GLP-1. a, GLP-1 concentrations in the culture supernatant of either $GIFT_{GLP-1}$ cells or constitutive GLP-1 constructs driven by different promoters. Cells were induced with 15 mM glucose for 12 h, and GLP-1 levels were measured by ELISA. **b**, Time schedule for oral administration of $GIFT_{GLP-1}$ or constitutive GLP-1 expression cells for glycemic control in db/db mice. $GIFT_{GLP-1}$ cells or constitutive GLP-1 constructs driven by different promoters were administered to db/db mice via gavage at a dose of 10^9 CFU. **c**, Blood glucose levels (fasting for 4 hours) were quantified using a glucometer 6 hours after gavage. **d**, Schematic of the time schedule for sub-culturing the engineered strains. $GIFT_{GLP-1}$ cells and EcN transformed with a plasmid for constitutive GLP-1 expression driven by a P_{tac} promoter (EcN-P_{tac}-GLP-1) were cultured in LB medium at 37°C, with a 1% inoculum sub-cultured every 12 h. **e**, ELISA for GLP-1 secreted by EcN-P_{tac}-GLP-1 and $GIFT_{GLP-1}$ cells across different generations. EcN-P_{tac}-GLP-1 cells and $GIFT_{GLP-1}$ cells from the 3rd, 6th, and 9th subcultures were induced with

15 mM glucose for 8 h, and the culture supernatant was collected for ELISA analysis. **f**, Schematic of the time schedule for detection of the biological activity of GLP-1 produced by EcN-P_{tac}-GLP-1 and $GIFT_{GLP\text{-}1}$ cells. EcN-P_{tac}-GLP-1 cells and $GIFT_{GLP\text{-}1}$ cells from the 3rd, 6th, and 9th subcultures were inducted with 15 mM glucose for 8 h, and the culture supernatant was supplemented to HEK293T cells expressing the GLP-1 receptor (GLP1R) and a SEAP reporter driven by a P_{CRE} promoter. g, SEAP production of HEK-293T cells treated with the culture supernatant of EcN-P_{tac}-GLP-1 and $GIFT_{GLP\text{-}1}$ cells. **h**, **i**, Blood glucose levels of db/db mice treated with EcN-P_{tac}-GLP-1 cells (**h**) and $GIFT_{GLP\text{-}1}$ cells (**i**) from the 3rd, 6th, and 9th subcultures. Data in **a, e,** and **g** are expressed as means ± SD; $n = 5$ independent experiments in **a**, $n = 3$ independent experiments in **e** and **g**. Data in **b, h,** and **i** are expressed as means ± SEM; $n = 5$ mice. *P*-values were calculated using one-way ANOVA (a) or two-way ANOVA (c, e, g-i) . NS, not significant; $***P < 0.001$.

[redacted]

[redacted]

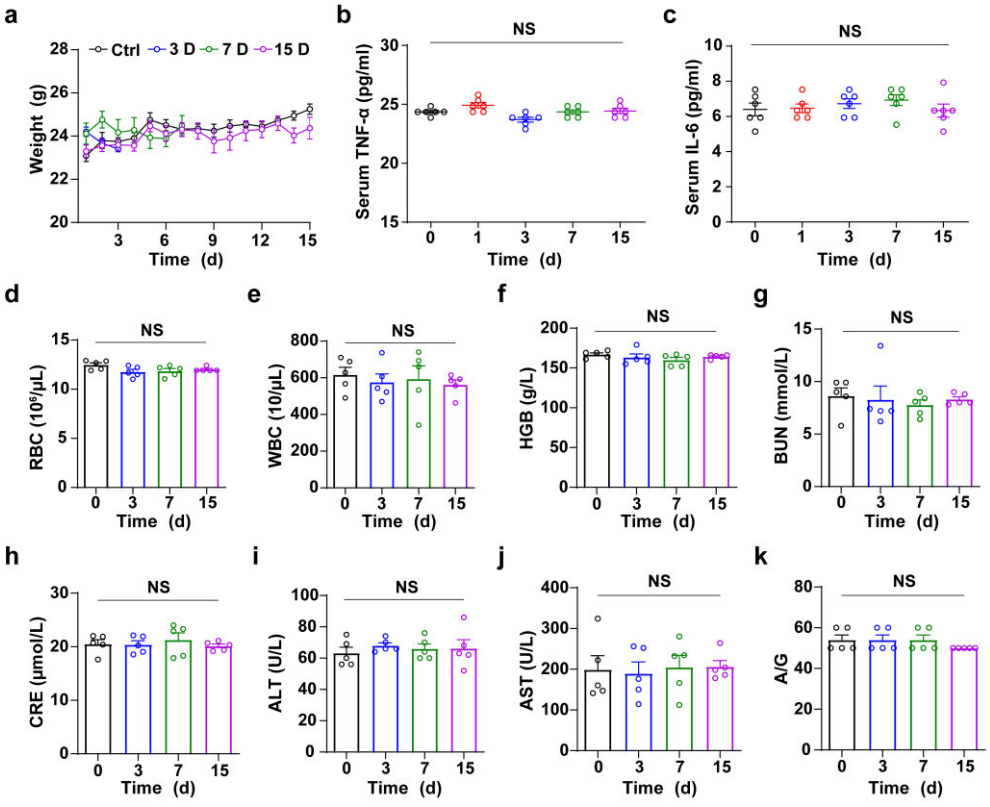
II. Regarding characterization of toxicity:

First, as demonstrated in our initial submission, we assessed the safety profile of the orally administered GIFT cells in mice, by monitoring body weight, cytokine levels, complete blood counts (CBC), and standard serum biochemical indices. To recap, no abnormalities were observed in the body weight of mice following oral administration of GIFT for any examined duration, and all cytokine, CBC, and serum biochemistry indexes indicated the absence of inflammation and the lack of hepatic/renal dysfunction (**Extended Data Fig. 4**). Also, we evaluated acute inflammatory and hypersensitivity responses in mice following a single administration of either $GIFT_{GLP\text{-}1}$ or semaglutide, as well as the risk of hypoglycemia when co-administered with insulin glargine. $GIFT_{GLP\text{-}1}$ demonstrated a superior safety profile and fewer behavioral side effects compared to semaglutide (**Extended Data Fig. 10a-n; Supplementary Tables 1–9**). Please also note that CBC and serum biochemistry analyses of type 2 diabetic monkeys treated with $GIFT_{GLP\text{-}1}$ revealed no significant abnormalities (**Supplementary Tables**

12–15). We interpreted these findings from the originally submitted manuscript as supporting a favorable safety and tolerability profile for GIFT cells.

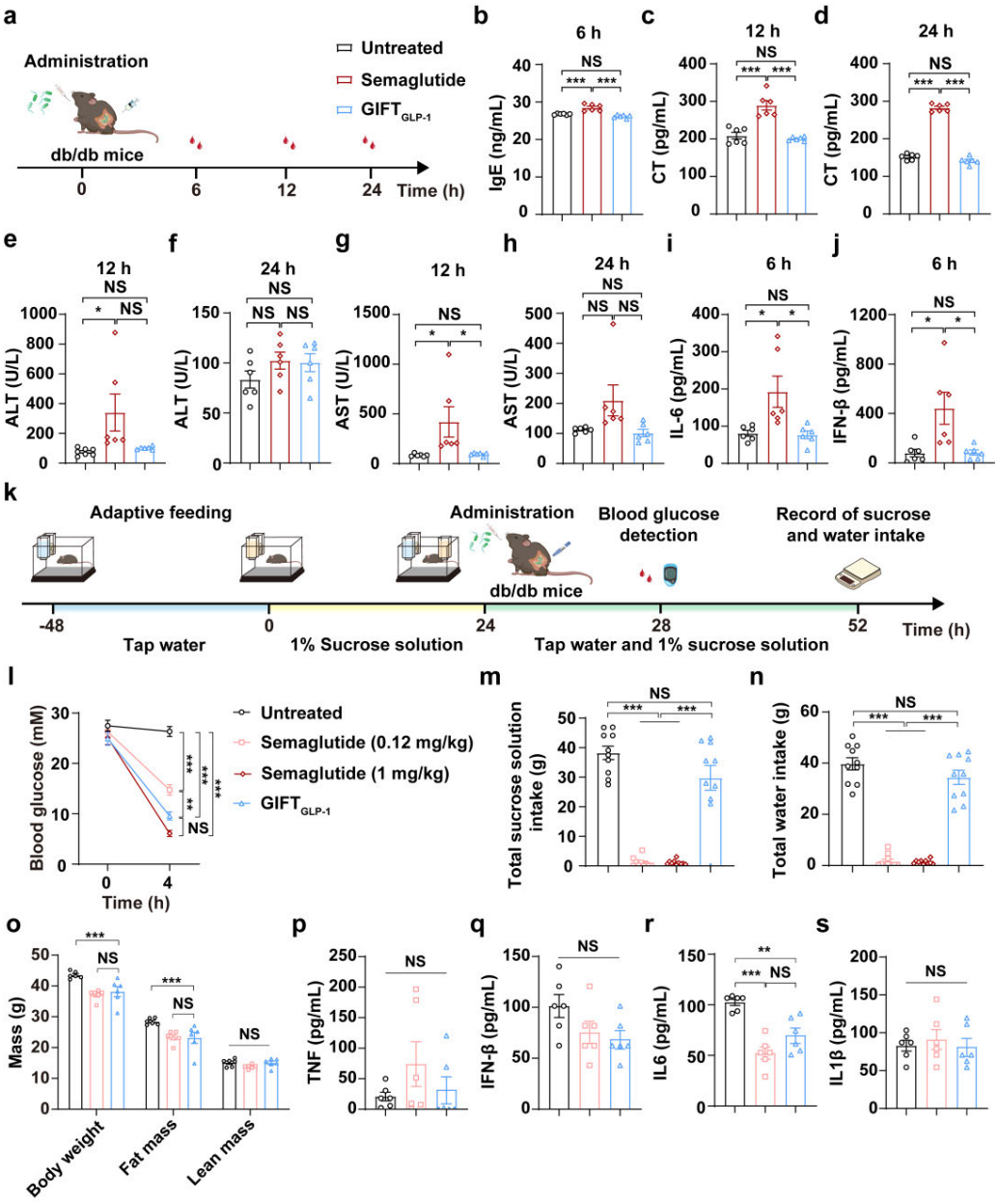
In the revised version, we have performed additional long-term toxicity studies comparing the two-month safety profile of $GIFT_{GLP\text{-}1}$ and semaglutide in db/db mice. We performed detailed analysis of the following parameters, including CBC (**new Supplementary Table 10**), serum biochemical profiles (**new Supplementary Table 11**), and quantification of proinflammatory cytokine levels (**new Extended Data Fig. 10p-s**). And we found that no significant differences were observed between the two treatment groups. Notably, for several parameters, $GIFT_{GLP\text{-}1}$ exhibited slightly better tolerability than semaglutide. These findings support the safety of our gut-based, orally delivered therapeutic system. These findings have been incorporated into our revised manuscript. Please see **page 17, lines 494-504** in the revised manuscript and below:

> "To evaluate the long-term safety of $GIFT_{GLP\text{-}1}$ cells, we conducted a comparative assessment in db/db mice treated with either $GIFT_{GLP\text{-}1}$ or semaglutide for two months. No significant differences were observed between the two groups in complete blood counts (**Supplementary Table 10**), serum biochemical parameters (**Supplementary Table 11**), or proinflammatory cytokine levels (**Extended Data Fig. 10p-s**), supporting the favorable safety profile of our gut-resident, orally delivered therapeutic system."



Extended Data Fig. 4: Assessment of the safety of GIFT after oral delivery to mice. a, Body weight of mice after gavage with 10^9 CFU of $GIFT_{GFP}$ for 3, 7, and 15 days. **b**, **c**, Cytokine abundance for TNF-α (**b**) and IL-6 (**c**) from the serum of treated mice. C57BL/6 mice were orally administrated with $GIFT_{GFP}$ (10^9 CFU/mouse/day) continuously for 1, 3, 7, and 15 days. Blood was collected at the indicated time points post-oral administration, and the cytokine abundance was quantified using ELISA kits. **d-k**, 24 hours after the final administration, the

blood of mice was collected for complete blood count and blood biochemical analysis. (**d**) Counts for total red blood cells (RBC), (**e**) white blood cells (WBC), and (**f**) hemoglobin (HGB). **g**, **h**, kidney function analysis, (**g**) blood urea nitrogen (BUN), (**h**) creatinine (CRE). **i-k**, Hepatic function analysis. (**i**) alanine aminotransferase (ALT), (**j**) aspartate aminotransferase (AST), (**k**) albumin/globulin ratio (A/G). Data are expressed as means ± SEM; *n* = 6 mice for **a-c**, *n* = 5 mice for **d-k**. *P*-values were calculated by one-way ANOVA. NS, not significant.



Extended Data Fig. 10: Comparative analysis of adverse effects between semaglutide injection and oral delivery of GIFT$_{GLP-1}$ in db/db mice. a, Schematic of the timeline for assessing acute adverse effects. db/db mice were divided into three groups: one received oral GIFT$_{GLP-1}$, another was injected with semaglutide, and the third group was left untreated. Blood samples were collected at 6 hours for serum immunoglobulin E (IgE), interleukin-6 (IL-6), and Interferon beta (IFN-β) analysis, and at 12 and 24 hours for complete blood count (CBC), serum biochemical analysis, and calcitonin (CT) measurement. **b**, Serum IgE levels in db/db mice 6

hours after treatment. **c-h**, Serum levels of CT (**c**, **d**), alanine transaminase (ALT) (**e**, **f**), and aspartate transaminase (AST) (**g**, **h**) in db/db mice 12 and 24 hours after treatment. **i**, **j**, Serum levels of IL-6 (**i**) and IFN-β (**j**) in db/db mice 6 hours after treatment. **k**, Schematic of the timeline for the conditioned taste aversion (CTA) experiment. db/db mice were single-housed with *ad libitum* access to chow diet and tap water for 48 hours, followed by access to 1% sucrose for 24 hours. After acclimation, mice given oral $GIFT_{GLP\text{-}1}$ or injected with semaglutide underwent a two-bottle preference test, choosing between tap water and 1% sucrose. Sucrose and water intake were recorded for 24 hours. **l**, Blood glucose levels monitored 4 hours after $GIFT_{GLP\text{-}1}$ or semaglutide administration. **m**, Total sucrose intake. **n**, Total water intake. **o**, Body weight, fat mass, and lean mass in db/db mice treated with semaglutide (0.12 mg/kg/week, intramuscular injection), $GIFT_{GLP\text{-}1}$ (10^9 CFU/day, oral administration), or were left untreated for one month. **p-s**, Serum levels of TNF (**p**), IFN-β (**q**), IL-6 (**r**), and IL-1β (**s**) in db/db mice after two-month treatment with semaglutide (0.12 mg/kg/week, intramuscular injection), $GIFT_{GLP\text{-}1}$ (10^9 CFU/day, oral administration), or on treatment. Data in **b-j** and **l-s** are expressed as means ± SEM; $n = 6$ mice in **b-j** and **o-s**, $n = 9$ mice in **l-n**. P-values were calculated using one-way ANOVA. NS, not significant; $^*P < 0.05$, $^{**}P < 0.01$, $^{**}P < 0.001$.

Supplementary Table 10: Complete blood count (CBC) in db/db mice after oral gavage of $GIFT_{GLP-1}$ cells (10^9 CFU/mouse/day) or injection of semaglutide (0.12 mg/kg/week) for two months.

Index	Wild-type mice	db/db mice		
		Untreated	Semaglutide	$GIFT_{GLP-1}$
WBC (10^3/μL)	5.78±0.77	5.07±1.06	4.18±0.86	5.97±1.09
RBC (10^6/μL)	10.67±0.16	12.35±0.74	12.79±0.89	12.86±0.98
HGB (g/dL)	16.05±0.22	17.58±1.04	18.83±1.4	18.88±1.45
HCT (%)	51.94±0.57	55.55±1.8	61.81±5.08	59.08±4.5
MCV (fL)	48.69±0.34	45.03±1.63	48.31±1.55	45.93±1.34
MCH (pg)	15.03±0.06	14.23±0.18	14.71±0.45	14.68±0.36
MCHC (g/dL)	30.90±0.14	31.63±1.19	30.48±0.4	31.95±0.38
RDW-SD (fL)	29.69±0.87	33.68±2.7	35.08±2.62	32.33±2.22
RDW-CV (%)	21.07±0.19	21.93±2.06	18.26±0.8	17.61±0.73
PDW (fL)	7.20±0.06	7.25±0.64	6.3±0.62	6.16±0.77
MPV (fL)	6.51±0.04	6.8±0.22	7.33±0.18	7.25±0.24
P-LCR (%)	4.25±0.15	3.93±0.9	5.9±0.92	4.76±1.19
PCT (%)	0.99±0.04	1.11±0.36	1.73±0.25	1.11±0.34
NEU (10^3/μL)	0.93±0.29	2.88±0.95	2.16±0.44	2.34±0.36
LYM (10^3/μL)	4.93±0.66	1.93±0.43	1.81±0.66	3.34±0.58
MON (10^3/μL)	0.19±0.04	0.21±0.07	0.11±0.04	0.24±0.14
EOS (10^3/μL)	0.04±0.01	0.04±0.02	0.09±0.19	0.03±0.03
NEU (%)	13.74±2.77	56.1±8.91	52.88±12.9	39.4±2.01
LYM (%)	82.49±3.01	38.93±9	42.28±11.39	56.06±1.67
MON (%)	3.03±0.42	4.1±1.26	2.56±0.94	3.91±1.78
EOS (%)	0.69±0.08	0.81±0.51	2.11±4.45	0.51±0.49
BAS (%)	0.10±0.04	0.05±0.08	0.15±0.12	0.1±0.08
RET (%)	4.78±0.34	3.62±0.8	3.25±0.54	2.79±0.52
RET (10^6/μL)	0.51±0.03	0.44±0.1	0.41±0.08	0.36±0.07
IRF (%)	56.27±0.71	53.98±3.78	60.51±4.35	53.73±5.42
LFR (%)	43.73±0.71	46.01±3.78	39.48±4.35	46.26±5.42
MFR (%)	19.88±0.79	14.73±1.99	12.36±1.2	14.86±1.45
HFR (%)	36.39±1.31	39.25±2.8	48.15±5.04	38.86±5.1
RET-He (pg)	16.67±0.24	16.7±0.35	17.81±0.84	16.83±0.25
IPF (%)	0.08±0.08	0.23±0.08	0.85±0.66	0.3±0.14
PLT-O (10^3/μL)	1029.70±39.57	1098±336.95	1481.33±214.85	1075.83±408.32
PLT-I (10^3/μL)	1516.00±64.39	1635.33±554.86	2355.5±375.39	1549.66±515.92
PLT-F (10^3/μL)	1702.67±629.05	1141.66±257.52	1630.83±207.16	1202.33±400.34
RBC-O (10^6/μL)	9.69±0.13	11.91±1.42	13.01±0.73	13.2±0.93

Abbreviations: WBC, white cell count; **RBC**, red cell count; **HGB**, hemoglobin; **HCT**, hemoglobin; **MCV**, mean corpusular volume; **MCH**, mean corpusular volume; **MCHC**, mean corpusular volume; **RDW**, mean corpusular volume; **PDW**, mean platelet volume; **MPV**, mean platelet volume; **P-LCR**, platelet -larger cell ratio; **PCT**, mean platelet volume; **NEU**, neutrophils; **LYM**, lymphocytes; **MON**, monocytes; **EOS**, eosinophils; **BAS**, basophils; **RET**, reticulocyte; **IRF**, immature reticulocyte fraction; **LFR**, low fluorescence reticulocyte; **MFR**, medium fluorescence reticulocyte; **HFR**, high fluorescence reticulocyte; **RET-He**, reticulocyte hemoglobin equivalent; **IPF**, immature platelet fraction; **PLT-O**, optical platelet cCount; **PLT-I**, impedance platelet count; **PLT-F**, fluorescent platelet count; **RBC-O**, optical red cell count.

Supplementary Table 11: Serum biochemistry analysis in db/db mice after oral gavage of $GIFT_{GLP-1}$ cells (10^9 CFU/mouse/day) or injection of semaglutide (0.12 mg/kg/week) for two months.

Index	Wild-type mice	db/db mice		
		Untreated	Semaglutide	$GIFT_{GLP-1}$
ALT (U/L)	36.11±3.80	314.75±99.05	136.75±48.34	194.78±35.9
AST (U/L)	126.11±10.23	441.04±92.75	256.67±62.41	345.82±55.64
UREA (mM)	10.62±1.35	14.25±2.69	13.24±2.43	12.6±1.1
CK (U/L)	1388.33±124.63	2214.36±519.76	1719.33±596.59	1785.7±840.36
LDH (U/L)	410.00±34.93	2838.61±414.29	2380.23±293.66	2345.31±116.03

Abbreviations: **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **CK**, creatine Kinase; **LDH**, lactate dehydrogenase.

III. Regarding the utility of a glucose-sensitive system:

Our rationale for implementing a glucose-sensitive system was to achieve real-time, feedback-controlled GLP-1 release in response to physiological blood glucose fluctuations, thereby mimicking the body's natural glycemic regulation more closely than conventional GLP-1 receptor agonists, which are clinically effective but are administered at fixed doses and act independently of real-time blood glucose levels. This lack of adaptive regulation may increase the risk of hypoglycemia during fasting or normoglycemia (*Mol Metab* 2021, PMID: 33068776; *Rev Diabet Stud* 2014, PMID: 26177483), especially when combined with insulin or sulfonylureas, and may also result in suboptimal postprandial glycemic control as the absence of physiologically synchronized hormone release (*Mol Metab* 2021, PMID: 33068776; *Rev Diabet Stud* 2014, PMID: 26177483). Further, numerous studies and clinical trials have highlighted a range of adverse effects associated with sustained high dosages of GLP-1 (*e.g.*, the systemic administration of GLP-1 receptor agonists), such as gastrointestinal discomfort, pancreatitis, pancreatic cancer, and thyroid complications, among others (*JAMA* 2023, PMID: 37796527; *Diabetes* 2012, PMID: 22266668; *Gastroenterology* 2011, PMID: 21334333; *J Clin Endocrinol Metab* 2012, PMID: 22031513).

Our engineered GIFT system addresses this limitation by autonomously sensing glucose and adjusting GLP-1 production in a responsive manner. This self-regulating mechanism enhances therapeutic safety and efficacy, and aligns with ongoing, broad shift in diabetes care toward context-aware and personalized interventions (*ACS Pharmacol Transl Sci* 2024, PMID: 38751616). The recent spotlight on glucose-sensitive insulin—named one of *Nature*'s top scientific breakthroughs of 2024—underscores the growing demand for adaptive therapeutic strategies that respond to internal physiological cues (*Nature* 2024, PMID: 39415004).

Another conceptual perspective may also be useful here: GIFT can be considered as a **"microbial incretin mimic"**, engineered to emulate intestinal L-cells' glucose-responsive GLP-1 secretion. Like native intestinal endocrine cells, GIFT dynamically monitors blood glucose and adjusts GLP-1 output accordingly, enabling biomimetic glycemic control without requiring external intervention.

Beyond the aforementioned advantages, GIFT offers a non-invasive and patient-

friendly delivery route, in theory eliminating the need for frequent injections, which would be expected to enhance long-term treatment adherence.

We have further summarized the utility of a glucose-sensitive system in the Discussion section of the revised manuscript. Please see **page 20, lines 562-573** and below:

> "Several efforts have been made to develop oral-engineered bacteria for GLP-1 expression, showing therapeutic potential in diabetes and obesity in mouse models[73,74]. However, constitutive expression often leads to low protein levels, imposes a metabolic burden on host bacteria[75], and lacks the ability for real-time on-demand release, compromising protein stability and clinical effectiveness. Using a HexR-based glucose sensor in GIFT cells allows for precise profiling of physiological blood glucose concentrations reversibly and dynamically. GIFT can be considered as a "microbial incretin mimic", engineered to emulate the glucose-sensing and GLP-1 secretion functions of intestinal L-cells. Like native intestinal endocrine cells, GIFT dynamically monitors glycemic fluctuations and modulates therapeutic GLP-1 output accordingly. This biomimetic control mechanism enables precise glycemic regulation while minimizing the systemic side effects commonly associated with continuous GLP-1 administration."

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* The secretion mechanism is unclear; The GLP produced by *E. coli* has periplasmic tags, which does not directly result in extracellular secretion and it is unclear how efficient in terms of % secretion; It is not clearly necessary in their data as many bacteria will naturally lyse and release payloads.

Reply:

We appreciate this opportunity to clarify the mechanism of GLP-1 secretion in GIFT cells and to address concerns regarding the use of periplasmic signal peptides, secretion efficiency, and the contribution of bacterial lysis.

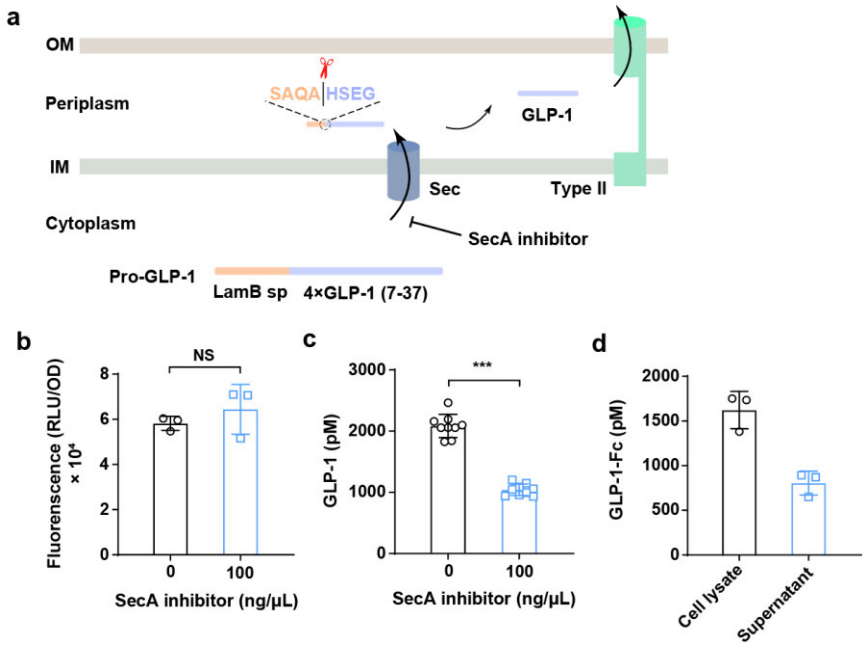
I. Secretion Mechanism and Use of the LamB Signal Peptide

In GIFT cells, GLP-1 is secreted mediated by the LamB signal peptide, which directs the protein to the periplasmic space before it is released into the extracellular environment (**Supplementary Fig. 5a**). The LamB signal peptide is a well-established tool in *E. coli* for protein secretion, facilitating the translocation of proteins across the inner membrane via the Sec-dependent secretion pathway (*J Bacteriol* 2003, PMID: 13129940; *J Bacteriol* 2012, PMID: 22056930). Specifically, the LamB signal peptide guides GLP-1 to the Sec translocon, where it is translocated across the inner membrane into the periplasmic space. Once in the periplasm, the signal peptide is cleaved off, and the mature GLP-1 protein is prepared for secretion (*Microbiol Spectr* 2016, PMID: 26999395). Subsequently, the type II secretion system facilitates the transport of GLP-1 from the periplasm to the extracellular environment (*Protein J* 2019, PMID: 30684070; *Recent Pat Biotechnol* 2010, PMID: 20201800). The use of LamB signal peptide reduces the reliance on bacterial lysis, enabling efficient secretion of GLP-1 into the supernatant.

We have conducted an additional experiment to assess the participation of the LamB signal peptide in GLP-1 secretion. We treated $GIFT_{GFP}$ and $GIFT_{GLP-1}$ cells with a Sec system inhibitor (SecA inhibitor) and measured GLP-1 levels in both cell lysates and supernatants (**Supplementary Fig. 5b, c**). The levels of GLP-1 were significantly reduced in the supernatant after inhibitor treatment, supporting that GLP-1 secretion is mediated by the LamB signal peptide.

As suggested by the referee, we quantified the secretion efficiency of GLP-1. ELISA analysis revealed that approximately 33.3% of the total GLP-1 produced was secreted into the supernatant in the presence of the LamB signal peptide (**Supplementary Fig. 5d**). In *E. coli*, heterologous peptide secretion is generally constrained, with Sec-dependent pathways typically yielding extracellular secretion efficiencies of ~20-40% (*J Biotechnol* 2010, PMID: 19958803; *Microb Cell Fact* 2018,

PMID: 30577801), depending on factors such as protein size, signal sequence, and expression level (*Appl Microbiol Biotechnol* 2004, PMID: 14966662). Thus, the observed ~33.3% secretion efficiency in our system falls within the upper range for *E. coli*-based secretion platforms, supporting the effective export of bioactive GLP-1.



Supplementary Fig. 5a-d: a, Schematic illustration of GLP-1 secretion by $GIFT_{GLP-1}$ cells mediated by the LamB signal peptide. Upon glucose induction, $GIFT_{GLP-1}$ cells express a Pro-GLP-1 construct [LamB-4×GLP-1(7-37)], which is translocated across the inner membrane (IM) via the Sec-dependent secretion pathway—a process that can be inhibited by SecA inhibitors. Within the periplasm, the LamB signal peptide (sp) is cleaved, and the mature GLP-1 fragment (residues 7-37) is subsequently transported across the outer membrane (OM) via the type II secretion system into the extracellular environment. **b**, **c**, Effect of SecA inhibition on protein expression and secretion by GIFT cells. $GIFT_{GFP}$ and $GIFT_{GLP-1}$ cells (10^9 CFU, 1 mL) were incubated with 15 mM glucose in the presence or absence of a SecA inhibitor (100 ng/μL) for 12 h. The fluorescence intensity of $GIFT_{GFP}$ cells was quantified using a Synergy H1 hybrid multimode microplate reader (**b**), and GLP-1 levels in the culture supernatant of $GIFT_{GLP-1}$ cells were quantified using an ELISA kit (**c**). **d**, Quantification of GLP-1-Fc [GLP-1(7-37) fused to human IgG] levels in the supernatant and cell lysate of $GIFT_{GLP-1-Fc}$ (GIFT-medaited GLP-1-Fc secretion) cells. $GIFT_{GLP-1-Fc}$ cells (10^9 CFU, 1 mL) were incubated with 15 mM glucose for 12 h. GLP-1-Fc concentrations in both the culture supernatant and cell lysate were measured using a human IgG ELISA kit. Data are expressed as means ± SD; $n = 3$ independent experiments in **b** and **d**, $n = 9$ independent experiments in **c**. *P*-values were calculated using a two-tailed unpaired *t*-test. NS, not significant; ***$P < 0.001$.

II. Contribution of Bacterial Lysis to Protein Release

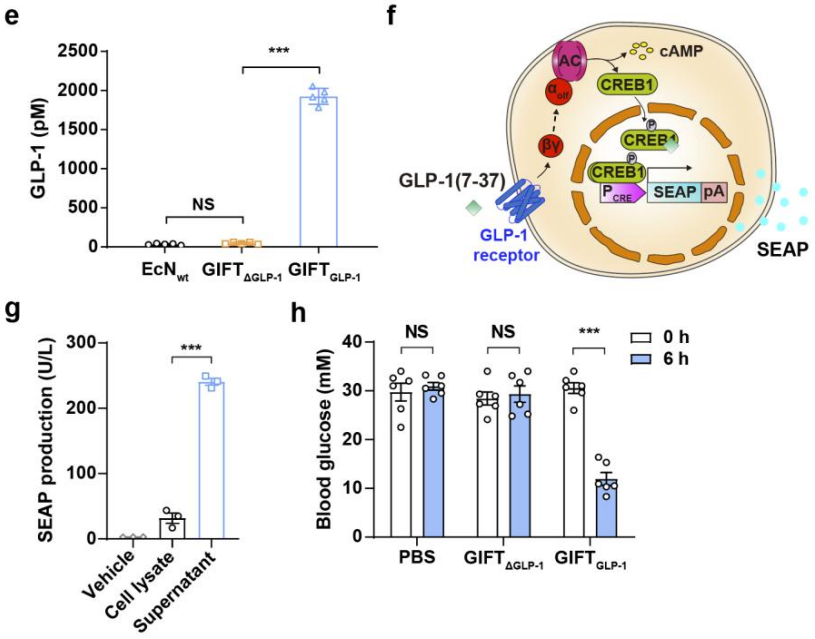
We acknowledge that spontaneous bacterial lysis may contribute to protein release; however, we found that bacterial lysis alone contributes minimally to GLP-1 secretion

in our GIFT$_{GLP-1}$ system. Specifically, we constructed a control strain expressing GLP-1 without any secretion signal peptide and compared extracellular GLP-1 levels. As shown in **Supplementary Fig. 5e**, only a minimal amount of GLP-1 was detected in the culture supernatant of the non-secretory strain. Moreover, oral administration of these bacteria did not result in a significant reduction in blood glucose levels in db/db mice (**Supplementary Fig. 5h**), indicating that lysis-derived GLP-1 plays an only marginal role in the elevated GLP-1 levels observed with GIFT$_{GLP-1}$ system.

GIFT achieves controlled GLP-1 release by incorporating secretion signal peptides that are **i.** functionally active in live cells and **ii.** responsive to glucose stimulation; it does not rely on spontaneous bacterial lysis. It is worth noting that the GLP-1 produced in our system is biologically active, as the signal peptide is cleaved during secretion to yield the mature fragment (residues 7-37); this is the therapeutically relevant form in humans (*Mol Metab* 2019, PMID: 31767182). In contrast, GLP-1 released through bacterial lysis retains the uncleaved signal peptide preventing the exposure of key GLP-1 receptor-activating residue (His7) (*Br J Pharmacol* 2012, PMID: 21950636; *Diabetologia* 2019, PMID: 31089754), thereby resulting in a loss of biological activity (**Supplementary Fig. 5f, g**).

The engineered secretion pathway is essential for both the secretion and functional maturation of GLP-1. These findings have been incorporated into our revised manuscript. Please see **page 9, lines 245-250** in the revised manuscript and below:

> "GLP-1 secretion was facilitated by the LamB signal peptide[48], resulting in approximately 33.3% of total GLP-1 detected in the culture supernatant (**Supplementary Fig. 5a-d**). In contrast, GLP-1 released through bacterial lysis exhibited impaired biological activity, likely due to retention of the uncleaved LamB signal peptide, which masks the key GLP-1 receptor-activating residue (His7)[49,50], thereby preventing formation of the mature active fragment (residues 7-37) (**Supplementary Fig. 5e-h**)."



Supplementary Fig. 5e-h: e, GLP-1 levels in the culture supernatant of wild-type EcN (EcN$_{wt}$)

and GIFT cells expressing GLP-1 with ($GIFT_{GLP-1}$) or without ($GIFT_{\Delta GLP-1}$) the LamB secretion signal peptide. Cells were induced with 15 mM glucose for 12 h, and the culture supernatant was collected for ELISA analysis. **f**, Schematic illustrating the detection of biologically active GLP-1 in culture supernatants or cell lysates from $GIFT_{GLP-1}$. Mature GLP-1(7-37) binds to and activates the GLP-1 receptor, triggering downstream signaling via cyclic AMP (cAMP). Elevated cAMP levels subsequently drive gene expression through the P_{CRE} promoter, resulting in measurable SEAP production. **g**, SEAP production of HEK-293T cells treated with the culture supernatant or cell lysate of $GIFT_{GLP-1}$. $GIFT_{GLP-1}$ cells (10^9 CFU) were incubated with 15 mM glucose for 12 h. HEK-293T cells transfected with the GLP-1 receptor (P_{SV40}-GLP1R-pA) and the corresponding reporter (P_{CRE}-SEAP-pA) were incubated with the culture supernatant or cell lysate for 24 h, and SEAP production was quantified. **h**, Blood glucose levels of db/db mice treated with PBS, $GIFT_{GLP-1}$, or $GIFT_{\Delta GLP-1}$. Data in **e**, **g** are expressed as means ± SD; $n = 5$ independent experiments in **e**, $n = 3$ independent experiments in **g**. Data in **h** are expressed as means ± SEM; $n = 6$ mice. *P*-values were calculated using a one-way ANOVA (**e**, **g**) or two-way ANOVA (**h**). NS, not significant; ***$P < 0.001$.

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* To evaluate primate data, the author should provide the blood glucose level of a regular monkey to compare the blood glucose level after dosing GIFT in a diabetic monkey.

Reply:

Thank you for this insightful suggestion. In response, we have now included comparative data on blood glucose levels in non-diabetic (healthy) monkeys, untreated diabetic monkeys, and GIFT-treated diabetic monkeys (**new Fig. 7h**). Following oral administration of $GIFT_{GLP\text{-}1}$ every three days, diabetic monkeys exhibited a sustained reduction in blood glucose of approximately 5 mM (~30%) over a one-month period. While blood glucose levels in GIFT-treated monkeys did not full return to the normoglycemic range observed in healthy controls, this extent of glycemic reduction is comparable to, and in some cases exceeds, outcomes reported for both conventional GLP-1 receptor agonists and cell-based GLP-1 delivery systems (*Sci Transl Med* 2019, PMID: 31645456; *Synth Syst Biotechnol* 2021, PMID: 34584995; *Endocrinology* 2018, PMID: 29992313). These results underscore the therapeutic potential of $GIFT_{GLP\text{-}1}$ in lowering blood glucose in diabetic primates and provide a meaningful benchmark by contextualizing the treatment effect relative to physiological glucose levels in healthy animals.

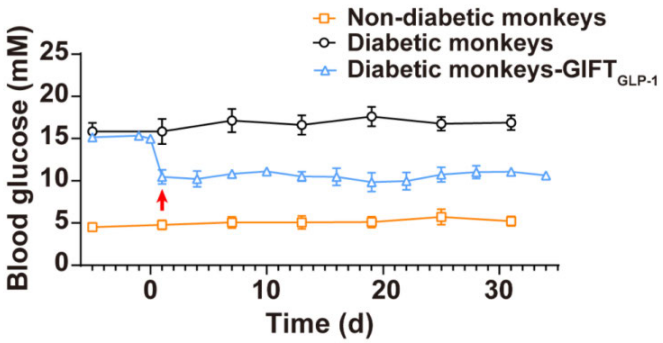

Fig. 7h: Fasting blood glucose levels in monkeys. Diabetic monkeys were orally administered $GIFT_{GLP\text{-}1}$ (2×10^{11} CFU/kg) every three days from Day 0 to Day 33. Fasting blood glucose levels were monitored every three days. The red arrow represents the time of the first administration. As controls, the blood glucose levels of non-diabetic monkeys and untreated diabetic monkeys were monitored every six days. Data are expressed as means ± SEM; $n = 5$ monkeys.

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* In the response document's Figure 1, how did the authors determine the concentration of the diverse carbon sources? For instance, 0.8% pyruvate (8 g/L) corresponds to approximately 90 mM, as shown in Figure 1a that they referred to, yet they opted to use 0.1 mM pyruvate for their experiment.

Reply:

Thank you for this question. The GIFT system developed in our study was specifically designed to sense and respond to physiologically relevant glucose concentrations in the gut. When evaluating the impact of various carbon sources on system activity, we selected concentrations that closely reflect their typical physiological levels in the human body.

For example, the serum concentration of succinate in healthy individuals have been reported at 43.93 ± 6.16 μM (*ISME J* 2018, PMID: 29434314). Plasma citrate levels in individuals with type 2 diabetes range from 104 to 147 μM (*J Clin Med* 2023, PMID: 37892807). The concentration of pyruvate in fasting adult venous and arterial blood is generally below 0.1 mM (*J Clin Pathol* 1962, PMID: 13928482). Lactate concentrations in the healthy adult gut are typically under 3 mM (*Appl Environ Microbiol* 2007, PMID: 17766450). Plasma glycerol concentrations approximate 0.05 mmol/L at rest, and rarely exceed 0.33–0.43 mM (*Sports Med* 1998, PMID: 9802172; *J Anal Toxicol* 2011, PMID: 22080901). Fasting systemic blood fructose concentrations are less than 0.05 mM, and in those healthy humans consuming high-fructose or sucrose diets, fructose concentrations are about 0.2-0.5 mM (*Nutrients* 2019, PMID: 31905727). Plasma free galactose concentrations in healthy individuals are typically below 0.05 mM (PMID: 20301691).

Although a concentration of 0.8% pyruvate (8 g/L), equivalent to ~90 mM, has been used in previous bacterial studies, we deliberately chose a more physiologically relevant concentration of 0.1 mM in our experiments to better reflect typical levels found in human tissues and fluids. Our goal was to assess the glucose-specific responsiveness of our system under biologically meaningful conditions.

We have included a summary of the reference concentrations for all tested carbon sources and their associated literature in the Methods of the revised manuscript. Please see **page 23, lines 646-652** and below:

> "To assess GIFT's responsiveness to a range of carbon sources, we selected concentrations that reflect physiological or pathophysiological levels reported in the human bloodstream or gastrointestinal tract. Specifically, we used 45 μM

succinate (43.93 ± 6.16 μM in obese individuals)[79], 0.2 mM citrate (104–147 μM in plasma from type 2 diabetes patients)[80], 0.1 mM pyruvate (<0.1 mM in fasting blood)[81], 3 mM lactate (<3 mM in the adult gut)[82], 0.15 mM glycerol (0.05–0.43 mM in plasma)[83,84], 0.1 mM fructose (0.05–0.5 mM in fasting systemic blood)[85], and 0.1 mM galactose (<0.05 mM in plasma)[86]."

References:

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* The 10% bioavailability mentioned on page 16 seems unclear. The concentration of bacteria in vitro (bacterial count per culture media volume) is not equivalent to the in vivo concentration (bacterial count in the gut relative to blood volume). Therefore, bioavailability should not be calculated simply as the in vivo concentration divided by the in vitro concentration.

Reply:

Thank you so much for the comments. In revised version, we have removed content about bioavailability from the revised text (see details, below). The traditional notion of bioavailability typically applies to small molecules or protein drugs. Thus, the concept of bioavailability may not be directly applicable to living therapeutics such as

the GIFT cells in our study.

The traditional definition of bioavailability—as the proportion of an administered drug that reaches systemic circulation in an active form—is applicable for drugs with well-defined pharmacokinetics, where systemic exposure is directly correlated with therapeutic efficacy. However, engineered living therapeutics like GIFT operate differently. Their activity is not determined by passive absorption or steady-state concentrations in plasma, but rather by their capacity to sense physiological cues (*e.g.*, elevated glucose), to respond dynamically by producing therapeutic proteins (*e.g.*, GLP-1), and to function locally—in our case within the gut.

Thus, applying classical pharmacokinetic parameters such as "bioavailability" to such systems is technically inappropriate. The therapeutic action of GIFT is driven by glucose-responsive expression of GLP-1, rather than systemic exposure to a fixed drug concentration. As such, we propose that the evaluation of GIFT cells should focus on functional outcomes such as dynamic responsiveness, blood glucose lowing efficacy, and safety, rather than conventional pharmacokinetic metrics.

Regarding the "10% bioavailability" reference on page 16, we acknowledge our error in using this term inappropriately. Our intention was to provide a rough estimation of the fraction of administered bacterial cells that might contribute to systemic GLP-1 effects, rather than to suggest a classic bioavailability value in pharmacological terms. We recognize that this phrasing was inappropriate, particularly given the differences between *in vitro* bacterial concentrations and the complex *in vivo* gastrointestinal environment.

In response to the referee's comment, we have removed all references to "bioavailability" from the revised manuscript. We now focus on the therapeutic efficacy and physiological performance of GIFT *in vivo*, which more accurately reflects the nature of this living therapeutic system.

****Addenda:**

The email we received from Dr. Burgess mentioned the following as a referee comment: "For referee 1, additional comments received separately include concerns about the effect sizes achieved, use of atypical IP injection route for glucose, and the degree of characterization/understanding of the system, such as the mechanism for the GLP-1 action in the gut". We would like to take this opportunity to address these points in detail.

Response to Referee's concern on effect sizes:

We appreciate the opportunity to clarify the effect size of our work.

In our study, the most relevant effect size was the glucose-lowering response resulting from dynamically regulated GLP-1 expression by the GIFT system. This was quantified as the percentage reduction in blood glucose over time following oral glucose administration. As shown in **Fig. 3**, GIFT treatment led to a ~40% reduction in blood glucose levels over 1 hour. Notably, the extent of glucose lowering was

comparable to that achieved by GLP-1 receptor agonists, such as semaglutide (**Extended Data Fig. 10l**), thereby demonstrating the therapeutic potential of our approach.

We further demonstrated a dose-dependent response: higher glucose concentrations led to stronger induction of GLP-1 expression and corresponding glucose reductions. Specifically, after administering $GIFT_{GLP\text{-}1}$ cells to db/db mice, GLP-1 expression was effectively induced by elevated blood glucose, peaking between 2 to 4 hours post-administration, and declining once glucose levels fell below 10 mM (**Fig. 3d, e**). Moreover, mice fed high-sugar foods such as cola and chocolate showed significantly lower blood glucose increase when treated with $GIFT_{GLP\text{-}1}$ cells compared to those receiving $GIFT_{Lux}$ cells (**Fig. 5g-k**), demonstrating the efficacy of $GIFT_{GLP\text{-}1}$ in modulating postprandial blood glucose fluctuations.

We would like to emphasize that the GIFT system is designed for adaptive regulation, rather than inducing a rapid or excessive drop in blood glucose. It provides continuous, real-time modulation to maintain glucose homeostasis. We demonstrated that oral administration of GIFT achieved 30 days of stable glycemic control in both mice and monkeys (**Fig. 3 and 7)**. Notably, compared to semaglutide, GIFT achieves an equivalent glucose-lowering effect while exhibiting lower incidences of hypersensitivity reactions (**Extended Data Fig. 10b),** reduced acute inflammatory responses (**Extended Data Fig. 10e-j)**, fewer adverse responses (**Extended Data Fig. 10m, n)**, and a reduced risk of hypoglycemia when co-administered with insulin glargine (**Supplementary Tables 3–9**). These findings support the consistent effects and safety of GIFT as a living therapeutic.

To further evaluate long-term safety and efficacy, we conducted an additional two-month study comparing GIFT cells with semaglutide in db/db mice. Complete blood counts (**Supplementary Table 10**), serum biochemistry profiles (**Supplementary Table 11**), and proinflammatory cytokine levels (**Extended Data Fig. 10p-s**) revealed no significant differences between the two groups, supporting the long-term safety and tolerability of GIFT.

In summary, GIFT achieves an excellent glucose-lowering effect through a biologically integrated, self-regulating mechanism, offering both sustained therapeutic efficacy and favorable safety. We view these results as a strong proof-of-concept for a next-generation living therapeutic platform.

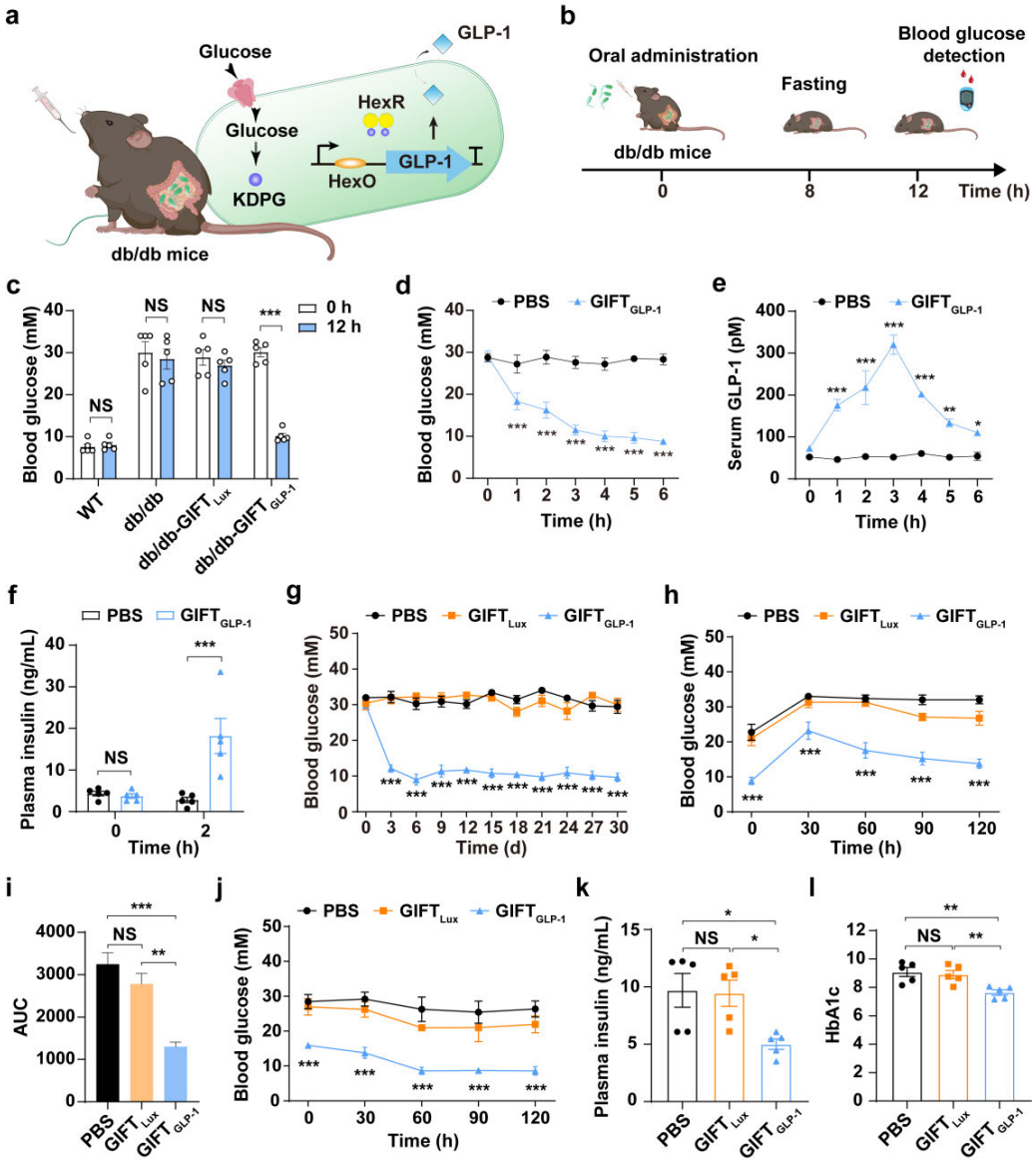
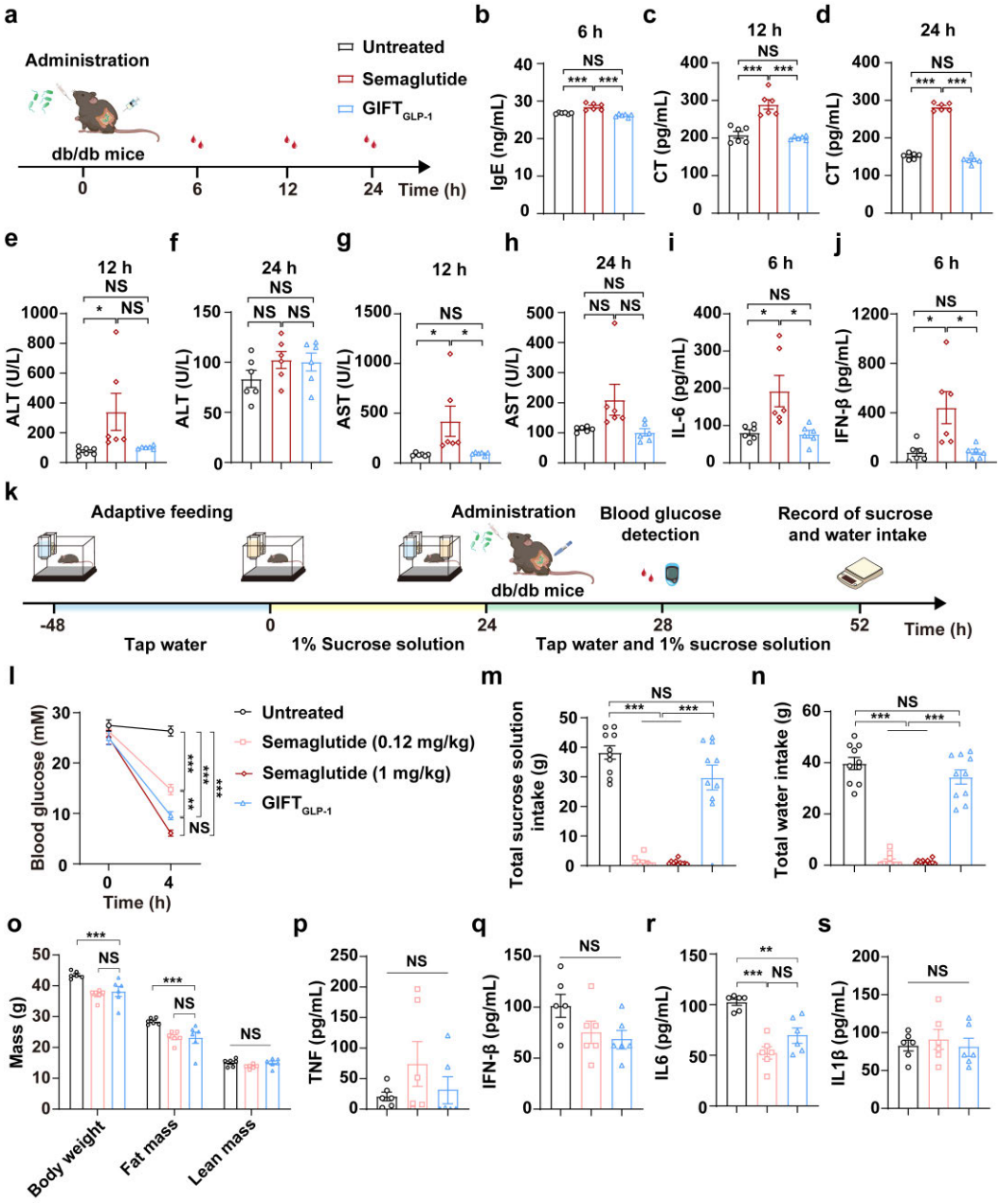


Fig. 3: Sense-and-response-based glycemic control in db/db mice. a, Schematic illustration of GIFT-mediated GLP-1 production in db/db mice. **b**, Time schedule for oral administration of GIFT cells expressing GLP ($GIFT_{GLP-1}$) for glycemic control in db/db mice. $GIFT_{Lux}$ or $GIFT_{GLP-1}$ cells were administered to wild-type and db/db mice via gavage at a dose of 10^9 CFU. **c**, Blood glucose levels (fasting for 4 hours) were quantified using a glucometer 12 hours after gavage. **d**, **e**, Changes in blood glucose and serum GLP-1 levels in db/db mice following oral administration of $GIFT_{GLP-1}$ cells. $GIFT_{GLP-1}$ cells (10^9 CFU) were administered via gavage, and blood glucose (**d**) and serum GLP-1 levels (**e**) were monitored over 6 hours. **f**, Plasma insulin levels in db/db mice, measured at 2 hours after oral administration of $GIFT_{GLP-1}$ cells. **g-l**, GIFT-mediated long-term glycemic control in db/db mice. PBS, $GIFT_{Lux}$, or $GIFT_{GLP-1}$ cells were orally administrated to db/db mice (10^9 CFU/mouse/day) for 30 days. Fasting blood glucose (**g**) was profiled every three days. Intraperitoneal glucose tolerance test (**h**) and insulin tolerance test (**j**) were performed on days 21 and 30, respectively. The average area under the curve in hours is presented in (**i**). Plasma insulin (**k**) and HbA1c levels (**l**) were analyzed 24 hours after the final gavage. Data in **c-l** are expressed as means ± SEM; n = 5 mice. P-values were

calculated using two-way ANOVA (**c-h** and **j**) or one-way ANOVA (**i**, **k**, and **l**). NS, not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.



Extended Data Fig. 10: Comparative analysis of adverse effects between semaglutide injection and oral delivery of GIFT$_{GLP-1}$ in db/db mice. a, Schematic of the timeline for assessing acute adverse effects. db/db mice were divided into three groups: one received oral GIFT$_{GLP-1}$, another was injected with semaglutide, and the third group was left untreated. Blood samples were collected at 6 hours for serum immunoglobulin E (IgE), interleukin-6 (IL-6), and Interferon beta (IFN-β) analysis, and at 12 and 24 hours for complete blood count (CBC), serum biochemical analysis, and calcitonin (CT) measurement. **b**, Serum IgE levels in db/db mice 6 hours after treatment. **c-h**, Serum levels of CT (**c**, **d**), alanine transaminase (ALT) (**e**, **f**), and aspartate transaminase (AST) (**g**, **h**) in db/db mice 12 and 24 hours after treatment. **i**, **j**, Serum

levels of IL-6 (**i**) and IFN-β (**j**) in db/db mice 6 hours after treatment. **k**, Schematic of the timeline for the conditioned taste aversion (CTA) experiment. db/db mice were single-housed with *ad libitum* access to chow diet and tap water for 48 hours, followed by access to 1% sucrose for 24 hours. After acclimation, mice given oral $GIFT_{GLP\text{-}1}$ or injected with semaglutide underwent a two-bottle preference test, choosing between tap water and 1% sucrose. Sucrose and water intake were recorded for 24 hours. **l**, Blood glucose levels monitored 4 hours after $GIFT_{GLP\text{-}1}$ or semaglutide administration. **m**, Total sucrose intake. **n**, Total water intake. **o**, Body weight, fat mass, and lean mass in db/db mice treated with semaglutide (0.12 mg/kg/week, intramuscular injection), $GIFT_{GLP\text{-}1}$ (10^9 CFU/day, oral administration), or were left untreated for one month. **p-s**, Serum levels of TNF (**p**), IFN-β (**q**), IL-6 (**r**), and IL-1β (**s**) in db/db mice after two-month treatment with semaglutide (0.12 mg/kg/week, intramuscular injection), $GIFT_{GLP\text{-}1}$ (10^9 CFU/day, oral administration), or on treatment. Data in **b-j** and **l-s** are expressed as means ± SEM; $n = 6$ mice in **b-j** and **o-s**, $n = 9$ mice in **l-n**. *P*-values were calculated using one-way ANOVA. NS, not significant; $*P < 0.05$, $**P < 0.01$, $**P < 0.001$.

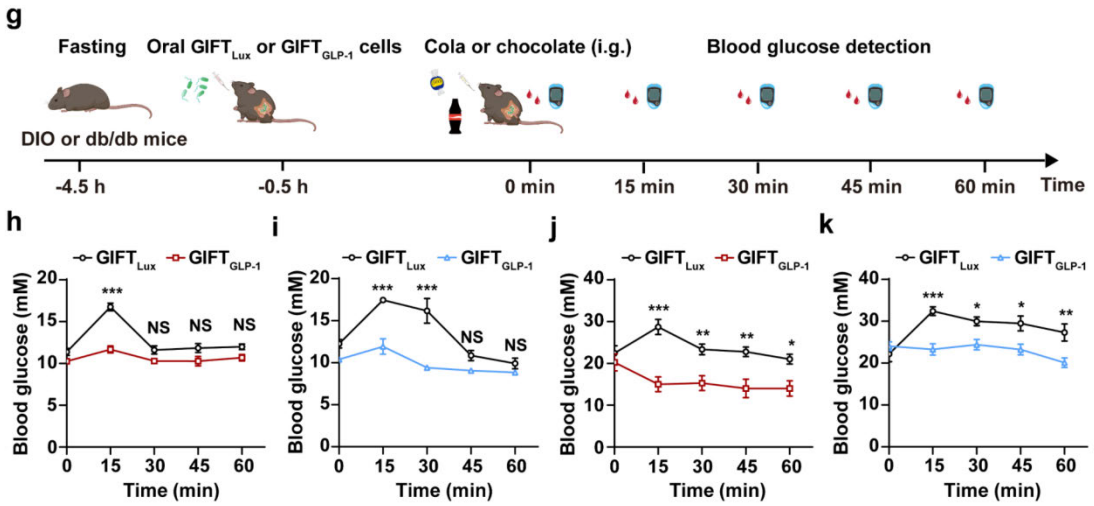


Fig. 5: GIFT-mediated postprandial glycemic control in high-fat diet-induced obese (DIO) mice and db/db mice. g, Time schedule for oral administration of $GIFT_{GLP\text{-}1}$ cells for postprandial glycemic control in DIO mice and db/db mice. After a 4-hour fasting period, mice were orally administered either $GIFT_{Lux}$ or $GIFT_{GLP\text{-}1}$ cells (10^9 CFU) and received cola or chocolate 30 min later. Blood glucose levels were monitored every 15 minutes. **h**, **i**, Blood glucose levels in DIO mice following intragastric administration of cola (**h**) or chocolate (**i**). **j**, **k**, Same as for **h**, **i**, but in db/db mice. Data in panels **h-k** are expressed as means ± SEM; $n = 6$ mice. *P*-values were calculated using two-way ANOVA. NS, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

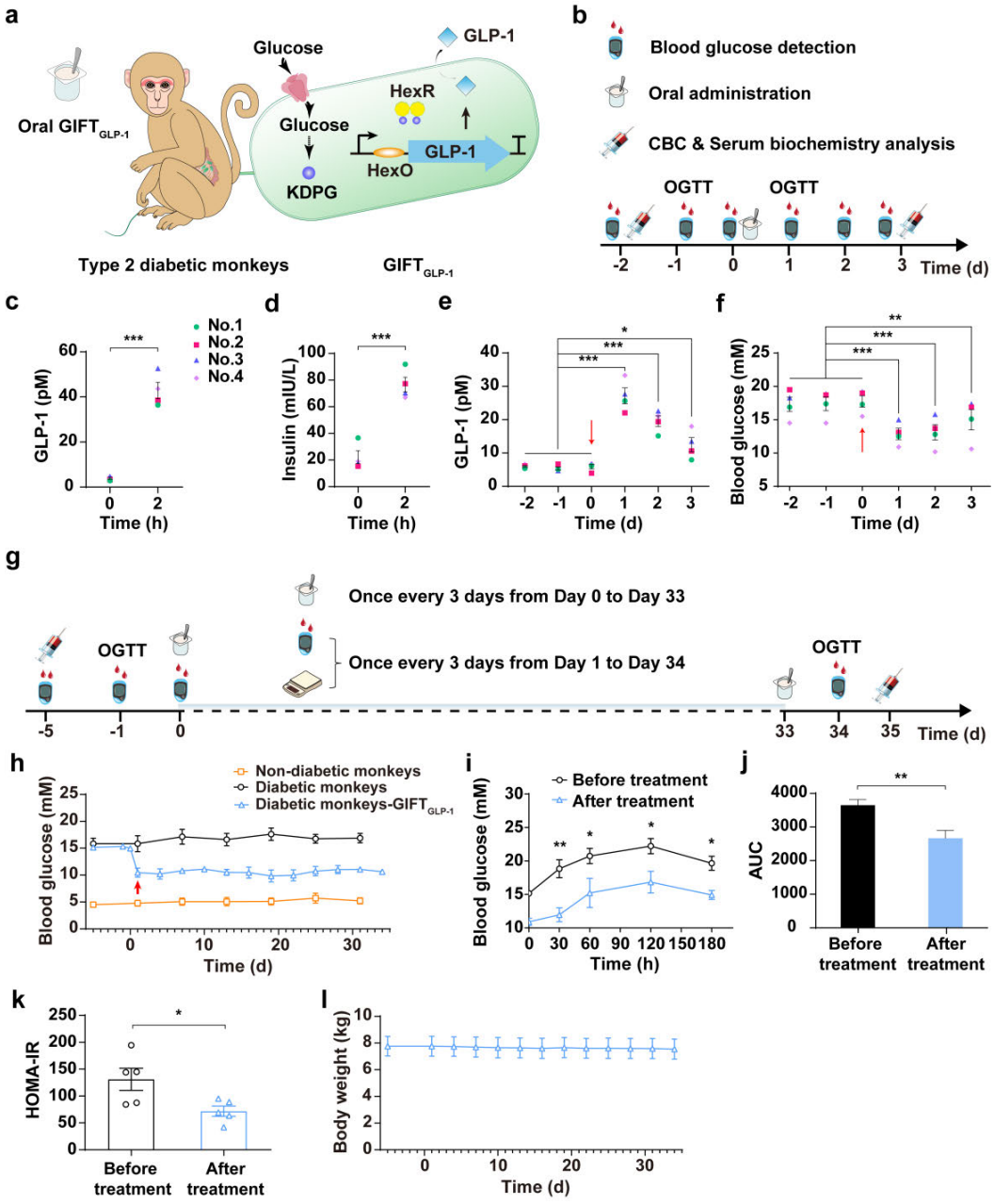


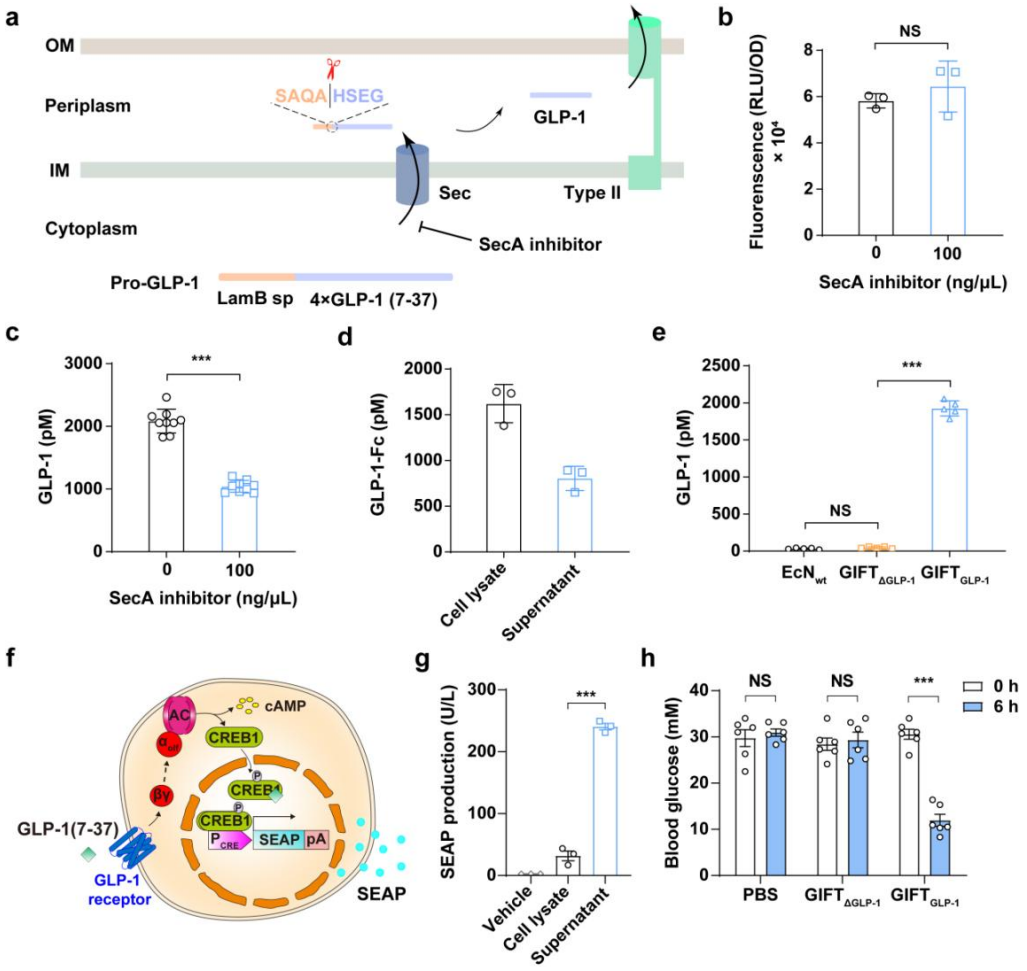
Fig. 7: Sense-and-response-based glycemic control in type 2 diabetic NHPs. a, Schematic for sense-and-response-based glycemic control in spontaneous type 2 diabetic monkeys. After oral administration, GIFT$_{GLP-1}$ cells produce GLP-1 in response to high glucose levels in the intestines of cynomolgus monkeys. **b**, Time schedule for oral administration of GIFT$_{GLP-1}$ for glycemic control in spontaneous type 2 diabetic monkeys following a single oral dose of GIFT$_{GLP-1}$. Four diabetic monkeys were orally administered GIFT$_{GLP-1}$ (2×10^{11} CFU/kg) on Day 0. Blood from four monkeys was collected for complete blood count (CBC) and serum biochemistry analysis on Day -2 and Day 3, respectively. **c**, Serum GLP-1 levels 2 hours post-administration. **d**, Plasma insulin levels 2 hours post-administration. **e**, **f**, Serum GLP-1 (**e**) and blood glucose (**f**) levels were monitored for 2 days before and 3 days after treatment. Red arrows represent the time of administration. **g**, Time schedule for long-term glycemic control in spontaneous type 2 diabetic monkeys. Five diabetic monkeys were orally administered GIFT$_{GLP-1}$ (2×10^{11} CFU/kg) every three days from Day 0 to Day 33. Blood glucose and body

weight were monitored every three days. Blood was collected for CBC and serum biochemistry analysis on Day -5 and Day 35. **h,** Fasting blood glucose levels. The red arrow represents the time of the first administration. As controls, the blood glucose levels of non-diabetic monkeys and untreated diabetic monkeys were monitored every six days. **i**, **j**, Oral glucose tolerance tests (OGTT) were conducted before (Day -1) and after treatment (Day 34). **k**, Insulin resistance was assessed before (Day -5) and after treatment (Day 35). **l,** Body weights of monkeys. Data in panels **c-f** and **h-l** are expressed as means ± SEM; $n = 4$ monkeys for **c-f**, and $n = 5$ monkeys for panels **h-l**. *P*-values were calculated by a two-tailed unpaired *t*-test (**c**, **d**, **j**, and **k**) or one-way ANOVA (**e**, **f**, and **i**). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Response to Referee's concern on GLP-1 mechanism in the gut:

In our study, the engineered GIFT cells are designed to sense elevated glucose levels in the intestinal lumen and dynamically express and secrete GLP-1. The GLP-1 peptide is secreted into the gut lumen via a dedicated bacterial secretion system (*i.e.*, rather than being released through bacterial lysis) (**Supplementary Fig. 5**). Once secreted, GLP-1 is absorbed across the intestinal epithelium into the portal or systemic circulation, consistent with the physiological behavior of endogenous GLP-1 produced by endocrine L cells (*Br J Pharmacol* 2022, PMID: 34235727) and previously reported pharmacokinetics of exogenous GLP-1 and GLP-1 receptor agonists (*Clin Pharmacol Ther* 2009, PMID: 19727071; *J Diabetes Sci Technol* 2012, PMID: 23294796; *Pharm Res* 2014, PMID: 24928365).

Following oral administration of $GIFT_{GLP-1}$ cells to db/db mice, we observed a rapid elevation in serum GLP-1 levels, peaking between 2 to 4 hours post-administration and subsequently declining as blood glucose levels dropped below 10 mM (**Fig 3d**, **e**). These findings suggest that the GLP-1 expressed by GIFT cells (i) is produced in a glucose-responsive manner and (ii) successfully transported across the intestinal barrier to enter the circulation and exert its glucose-lowering effects.



Supplementary Fig. 5. Effect of secretion signal peptide on GLP-1 secretion. a, Schematic illustration of GLP-1 secretion by $GIFT_{GLP-1}$ cells mediated by the LamB signal peptide. Upon glucose induction, $GIFT_{GLP-1}$ cells express a Pro-GLP-1 construct [LamB-4×GLP-1(7-37)], which is translocated across the inner membrane (IM) via the Sec-dependent secretion pathway—a process that can be inhibited by SecA inhibitors. Within the periplasm, the LamB signal peptide (sp) is cleaved, and the mature GLP-1 fragment (residues 7-37) is subsequently transported across the outer membrane (OM) via the type II secretion system into the extracellular environment. **b**, **c**, Effect of SecA inhibition on protein expression and secretion by GIFT cells. $GIFT_{GFP}$ and $GIFT_{GLP-1}$ cells (10^9 CFU) were incubated with 15 mM glucose in the presence or absence of a SecA inhibitor (100 ng/μL) for 12 h. The fluorescence intensity of $GIFT_{GFP}$ cells was quantified using a Synergy H1 hybrid multimode microplate reader (**b**), and GLP-1 levels in the culture supernatant of $GIFT_{GLP-1}$ cells were quantified using an ELISA kit (**c**). **d**, Quantification of GLP-1-Fc [GLP-1(7-37) fused to human IgG] levels in the supernatant and cell lysate of $GIFT_{GLP-1-Fc}$ (GIFT-medaited GLP-1-Fc secretion) cells. $GIFT_{GLP-1-Fc}$ cells (10^9 CFU, 1 mL) were incubated with 15 mM glucose for 12 h. GLP-1-Fc concentrations in both the culture supernatant and cell lysate were measured using a human IgG ELISA kit. **e**, GLP-1 levels in the culture supernatant of wild-type EcN (EcN_{wt}) and GIFT cells expressing GLP-1 with ($GIFT_{GLP-1}$) or without ($GIFT_{\Delta GLP-1}$) the LamB secretion signal peptide. Cells were induced with 15 mM glucose for 12 h, and the culture supernatant was collected for ELISA analysis. **f**, Schematic illustrating the detection of biologically active GLP-1 in culture

supernatants or cell lysates from $GIFT_{GLP\text{-}1}$. Mature GLP-1(7-37) binds to and activates the GLP-1 receptor, triggering downstream signaling via cyclic AMP (cAMP). Elevated cAMP levels subsequently drive gene expression through the P_{CRE} promoter, resulting in measurable SEAP production. **g**, SEAP production of HEK-293T cells treated with the culture supernatant or cell lysate of $GIFT_{GLP\text{-}1}$. $GIFT_{GLP\text{-}1}$ cells (10^9 CFU) were incubated with 15 mM glucose for 12 h. HEK-293T cells transfected with the GLP-1 receptor (P_{SV40}-GLP1R-pA) and the corresponding reporter (P_{CRE}-SEAP-pA) were incubated with the culture supernatant or cell lysate for 24 h, and SEAP production was quantified. **h**, Blood glucose levels of db/db mice treated with PBS, $GIFT_{GLP\text{-}1}$, or $GIFT_{\Delta GLP\text{-}1}$. Data in **b-e**, **g** are expressed as means ± SD; $n = 3$ independent experiments in **b**, **d**, and **g**, $n = 9$ independent experiments in **c**, $n = 5$ independent experiments in **e**. Data in **h** are expressed as means ± SEM; $n = 6$ mice. *P*-values were calculated using a two-tailed unpaired *t*-test (**b**, **c**), one-way ANOVA (**e**, **g**), or two-way ANOVA (**h**). NS, not significant; $***P < 0.001$.

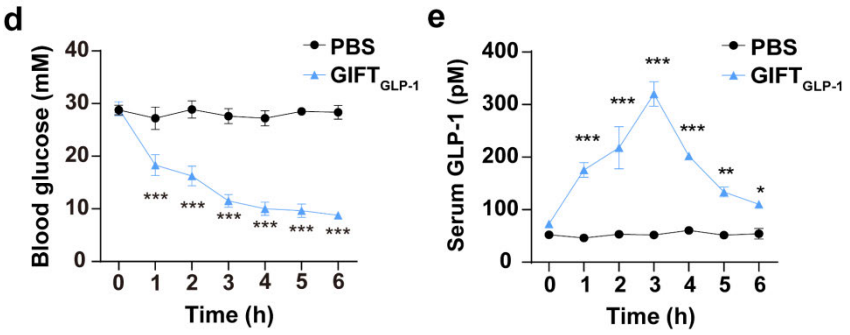


Fig. 3d, e: Changes in blood glucose and serum GLP-1 levels in db/db mice following oral administration of $GIFT_{GLP\text{-}1}$ cells. $GIFT_{GLP\text{-}1}$ cells (10^9 CFU) were administered via gavage, and blood glucose (**d**) and serum GLP-1 levels (**e**) were monitored over 6 hours. Data are expressed as means ± SEM; $n = 5$ mice. *P*-values were calculated using two-way ANOVA. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Response to Referee's concern on intraperitoneal glucose administration:

We now understand and appreciate the referee's concern that oral glucose administration better reflects physiological glucose dynamics compared to intraperitoneal (IP) injection. To address this, we have now completed an additional experiment in wild-type mice using oral glucose gavage to simulate blood glucose fluctuations. Specifically, wild-type mice harboring $GIFT_{GLP\text{-}1}$ cells were administered either a single oral dose of glucose (5g/kg) or two consecutive doses, with serum GLP-1 levels monitored over time. Mice receiving a single glucose gavage exhibited a transient increase in serum GLP-1 levels, which rapidly declined (within 60 min) (**Supplementary Fig. 9a-b**). In contrast, mice receiving two consecutive glucose gavages exhibited a prolonged elevation in serum GLP-1 levels (more than 90 min). These findings demonstrate that $GIFT_{GLP\text{-}1}$ cells secrete GLP-1 totally in a glucose-dependent manner *in vivo*. These findings have been incorporated into our revised

manuscript. Please see **page 13, lines 378-383** in the revised manuscript and below:

> "We also continuously monitored serum GLP-1 levels in wild-type mice harboring $GIFT_{GLP\text{-}1}$ cells following oral glucose administration. A single glucose gavage induced a transient increase in serum GLP-1, which rapidly returned to base line levels (**Supplementary Fig. 9a, b**). In contrast, two consecutive glucose gavages resulted in a prolonged elevation of serum GLP-1 levels, highlighting the dynamic responsiveness and reversibility of glucose-induced GLP-1 expression in our system in *vivo*."

We would also like to clarify that IP glucose administration was used in only one experiment in our study; that experiment was designed to simulate rapid and short-term changes (within one hour) in blood glucose levels in wild-type mice. To our understanding, this approach is well-established for modeling acute glucose responses (*Adv Mater* 2024, PMID: 38752744). It allowed us to capture the dynamics of glucose-responsive gene expression (ON-OFF-ON and OFF-ON-OFF) *in vivo* using the GIFT system (**Fig. 2f-k**).

We have multiple lines of *in vivo* evidence supporting the sensitive glucose responsiveness of GIFT:

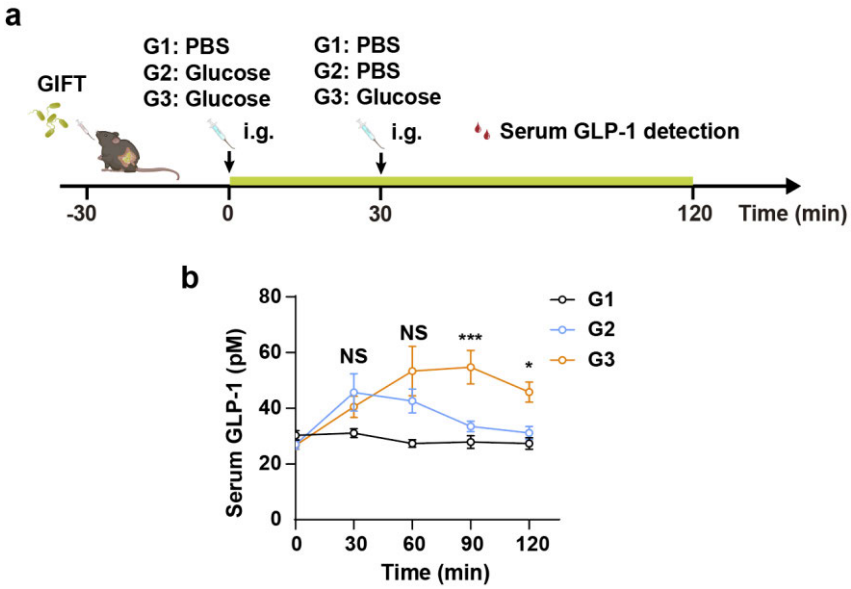
i) GIFT-mediated LuxCDABE expression was induced in db/db mice but not in wild-type mice (**Fig. 2l-n** and **Supplementary Fig. 4**), indicating that the system is activated under hyperglycemic conditions.

ii) Oral glucose induced LuxCDABE expression in wild-type mice carrying $GIFT_{Lux}$ cells, with expression levels increasing alongside glucose concentration (**Extended Data Fig. 9a-c** and **Supplementary Fig. 8**). These findings demonstrate that gene expression in GIFT cells is responsive to physiological glucose.

iii) In db/db mice treated with $GIFT_{GLP\text{-}1}$, serum GLP-1 level increased in response to elevated blood glucose; and the serum GLP-1 level decreased as the glucose level declined, demonstrating real-time feedback control (**Fig. 3d-e**).

iv) In wild-type, high-fat diet-induced obese (DIO), and db/db mice, oral high-sugar foods (*e.g.*, cola and chocolate) triggered rapid blood glucose spikes accompanied by LuxCDABE expression from $GIFT_{Lux}$ cells, whereas low-sugar foods did not (**Extended Data Fig. 9d-f** and **Supplementary Fig. 10-12**). Moreover, $GIFT_{GLP\text{-}1}$ administration significantly reduced the extent of postprandial blood glucose elevation in DIO and db/db mice (**Figure 5g-k**). These findings demonstrate the ability of the GIFT system to detect and respond to dietary glucose.

Taken together, these findings demonstrate the glucose responsiveness of GIFT *in vivo*, highlighting its advantages over constitutive expression systems in terms of responsiveness (**Fig. 2c-e**).



Supplementary Fig. 9: GIFT-mediated GLP-1 secretion kinetics in wild-type mice following oral glucose administration. a, Time schedule for GIFT-mediated GLP-1 secretion in wild-type mice following oral glucose administration. After a 4-hour fast, wild-type mice were orally administered $GIFT_{GLP\text{-}1}$ cells (10^9 CFU) and randomly divided into three groups. Thirty minutes later (defined as 0 min), mice in group 1 (G1) received an intragastric (i.g.) administration of PBS, while mice in group 2 (G2) and group 3 (G3) received oral glucose (5g/kg). At 30 min, G1 and G2 received PBS, while G3 was given a second oral glucose dose (5 g/kg). Serum GLP-1 levels were measured by ELISA every 30 min from 0-120 min. **b**, Serum GLP-1 levels of mice in different group. Data are expressed as means ± SEM; $n = 5$ mice. *P*-values were calculated using two-way ANOVA by comparing G2 with G3. NS, not significant; $*P < 0.05$, $***P < 0.001$.

References:

Holst JJ, Andersen DB, Grunddal KV. Actions of glucagon-like peptide-1 receptor ligands in the gut. ***Br J Pharmacol***. 2022.179(4):727-742. PMID: 34235727.

Steinert RE, Poller B, Castelli MC, Friedman K, Huber AR, Drewe J, Beglinger C. Orally administered glucagon-like peptide-1 affects glucose homeostasis following an oral glucose tolerance test in healthy male subjects. ***Clin Pharmacol Ther***. 2009.86(6):644-50. PMID: 19727071.

Araújo F, Fonte P, Santos HA, Sarmento B. Oral delivery of glucagon-like peptide-1 and analogs: alternatives for diabetes control? ***J Diabetes Sci Technol***. 2012.6(6):1486-97. PMID: 23294796.

Agarwal P, Khatri P, Billack B, Low WK, Shao J. Oral delivery of glucagon like peptide-1 by a recombinant Lactococcus lactis. ***Pharm Res***. 2014.31(12):3404-14. PMID: 24928365.

Bakhshandeh F, Zheng H, Barra NG, Sadeghzadeh S, Ausri I, Sen P, et al. Wearable Aptalyzer Integrates Microneedle and Electrochemical Sensing for In Vivo Monitoring of Glucose and Lactate in Live Animals. ***Adv Mater***. 2024.36(35):e2313743. PMID: 38752744.

Again, we express our sincere gratitude for the helpful guidance in improving our study and manuscript. Many thanks!

Referee #2 (Remarks to the Author):

The authors have responded to the questions posed in a thorough and comprehensive manner, conducting new experiments that the reviewer deems highly commendable. In their response, the authors indicate that they have adjusted the energy expenditure for lean mass. Please describe the methodology used to measure lean mass. It would be beneficial to include the lean mass, or preferably muscle mass, in the same Figure 7. Furthermore, the authors posit that the observed increase in energy expenditure in GIFT-GLP-1-treated db/db mice is largely attributable to their increased lean body mass (p. 40/51 in the rebuttal letter). This assertion requires further substantiation. One of the most commonly observed adverse effects of GLP-1 RA and weight loss in general is a reduction in lean mass, particularly muscle mass (*e.g.* PMID: 39401279). If this were not the case with GIFT, it would be a significant factor in further development. The authors should therefore include the development of lean mass in their models in Figure 7, particularly in the models in comparison with semaglutide.

Reply:

We sincerely thank the referee for the thoughtful and encouraging comments. We also appreciate the recognition of our additional experiments and responses.

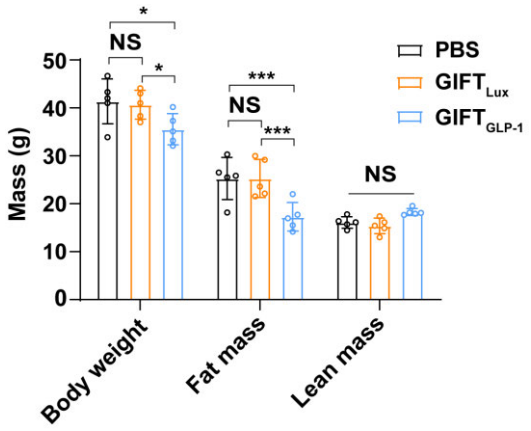
In response to the referee's concern regarding the methodology used to measure lean mass, we have added detailed information in the revised Methods section (**page 26, lines 757–760**). Specifically, fat mass and lean mass were measured using the AccuFat MRI system (AccuFat-1050, MAG-MED), a non-invasive analyzer based on magnetic resonance imaging (MRI) that is widely used for *in vivo* body composition analysis in live mouse (*Cell* 2022, PMID: 35247329).

We understand the Referee's specific point that GLP-1 receptor agonists, including semaglutide, are often associated with reductions in lean and muscle mass during weight loss. The loss of lean mass, particularly muscle mass, is a well-documented adverse effect of GLP-1-based therapies. However, our data suggest that this may not be the case with $GIFT_{GLP\text{-}1}$ treatment. In our initial experiments, we observed no significant decrease in lean mass in GIFT-treated mice (**new Extended Data Fig. 6d**).

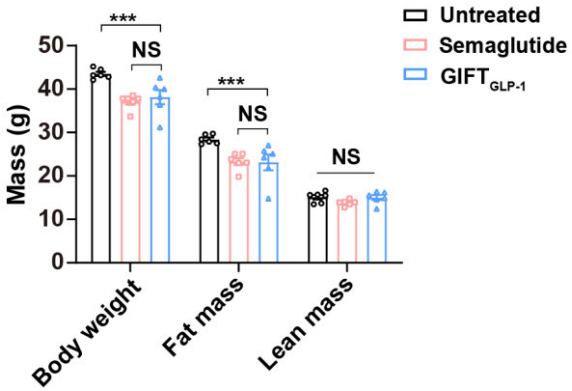
Further, we have conducted new experiments to directly compare the effects of $GIFT_{GLP\text{-}1}$ and semaglutide on lean mass. Specifically, db/db mice were treated with semaglutide (0.12 mg/kg/week, intramuscular injection), $GIFT_{GLP\text{-}1}$ (10^9 CFU/day, oral administration), or were left untreated for one month. Body weight, fat mass, and lean mass were measured at the end of the treatment period (**new Extended Data Fig. 10o**). Both the semaglutide and $GIFT_{GLP\text{-}1}$ treatments resulted in significantly reduced total body weight and fat mass. Neither treatment led to a statistically significant reduction in lean mass. Semaglutide-treated mice exhibited a trend toward lean mass reduction, consistent with previous reports (*Nat Metab* 2022, PMID: 35995995; *Biochim Biophys Acta Mol Basis Dis* 2024, PMID: 37793464), whereas $GIFT_{GLP\text{-}1}$-treated mice showed a modest increase in lean mass. Notably, several studies have likewise reported that semaglutide or other GLP-1 analogs did not significantly reduce lean mass reduction in diet-induced obesity or db/db mouse models (*JCI Insight* 2020, PMID: 32213703; *Front Physiol* 2019, PMID: 31404283; *Front Physiol* 2017, PMID: 28555111).

Taken together, these findings suggest that, unlike semaglutide, $GIFT_{GLP\text{-}1}$ may preserve lean mass during weight loss. This represents a potential therapeutic advantage of our system. One possible explanation is that the relatively high dose of semaglutide

may contribute to adverse effects on muscle mass. In contrast, $GIFT_{GLP\text{-}1}$ enables on-demand, glucose-responsive secretion of GLP-1, thereby avoiding sustained supraphysiological plasma GLP-1 levels. This regulated secretion may help alleviate catabolic effects on muscle and support the maintenance of lean mass.



Extended Data Fig. 6d: Body weight, fat mass, and lean mass in db/db mice with different treatment. db/db mice were treated with PBS, $GIFT_{Lux}$, or $GIFT_{GLP\text{-}1}$ (10^9 CFU/day) for one month. Body weight, fat mass, and lean mass were measured at the end of the treatment period. Data are expressed as means ± SEM; $n = 5$ mice. *P*-values were calculated using two-way ANOVA. NS, not significant; $*P < 0.05$, $**P < 0.001$.



Extended Data Fig. 10o: Body weight, fat mass, and lean mass in db/db mice with different treatment. db/db mice were treated with semaglutide (0.12 mg/kg/week, intramuscular injection), $GIFT_{GLP\text{-}1}$ (10^9 CFU/day, oral administration), or were left untreated for one month. Body weight, fat mass, and lean mass were measured at the end of the treatment period. Data are expressed as means ± SEM; $n = 6$ mice. *P*-values were calculated using two-way ANOVA. NS, not significant; $*P < 0.05$, $**P < 0.001$.

References:

Li Y, Wang D, Ping X, Zhang Y, Zhang T, Wang L, et al. Local hyperthermia therapy induces browning of white fat and treats obesity. ***Cell***. 2022.185(6):949-966.e19. PMID: 35247329.

Quarta C, Stemmer K, Novikoff A, Yang B, Klingelhuber F, Harger A, et al. GLP-1-mediated delivery of tesaglitazar improves obesity and glucose metabolism in male mice. ***Nat Metab***.

2022.4(8):1071-1083. PMID: 35995995.

Chen SY, Beretta M, Olzomer EM, Alexopoulos SJ, Shah DP, Byrne FL, et al. Head-to-head comparison of BAM15, semaglutide, rosiglitazone, NEN, and calorie restriction on metabolic physiology in female db/db mice. ***Biochim Biophys Acta Mol Basis Dis***. 2024.1870(1):166908. PMID: 37793464.

Gabery S, Salinas CG, Paulsen SJ, Ahnfelt-Rønne J, Alanentalo T, Baquero AF, et al. Semaglutide lowers body weight in rodents via distributed neural pathways. ***JCI Insight***. 2020.5(6):e133429. PMID: 32213703.

Ren L, Cui Q, Liu W, Wang L, Liao Y, Feng Y, et al. Novel GLP-1 analog supaglutide stimulates insulin secretion in mouse and human islet beta-cells and improves glucose homeostasis in diabetic mice. ***Front Physiol***. 2019.10:930. PMID: 31404283.

Wan Y, Bao X, Huang J, Zhang X, Liu W, Cui Q, et al. Novel GLP-1 analog supaglutide reduces hfd-induced obesity associated with increased ucp-1 in white adipose tissue in mice. ***Front Physiol***. 2017.8:294. PMID: 28555111.

Again, we express our sincere gratitude for the helpful guidance you provided in improving our study. Many thanks!

Nature 2023-10-18581B-Z: Point-by-point response

We would like to thank the Editor and all Referees for their constructive guidance, which has helped us improve our study. As guided by the Editor, and given the four-week revision timeframe, we addressed the remaining comments primarily through analysis of existing data, additional analyses of previously collected samples, and text-based clarification where new experiments were not feasible. Below, we provide point-by-point responses to each of the referee comments. We begin with a brief summary of the major updates and improvements made during the revision process.

1. We performed 16S rRNA gene sequencing to assess the impact of $GIFT_{GLP\text{-}1}$ on the gut microbiome of db/db mice after 30 days of treatment. Compared with PBS-treated controls, $GIFT_{GLP\text{-}1}$ treatment increased the relative abundance of the beneficial commensal *Faecalibacterium prausnitzii* while reducing levels of potentially pathogenic and inflammation-associated taxa, including *Enterococcus faecalis* and *Acetatifactor muris* (**Supplementary Fig. 8, 9**). These new results demonstrate that $GIFT_{GLP\text{-}1}$ has the capacity to beneficially restructure the gut microbial community.

2. We performed portal vein sampling after oral administration of GIFT and observed significantly higher GLP-1 levels in portal venous blood than in peripheral circulation (**Fig. 2g**), indicating a strong portal gradient. Thus, GIFT-derived GLP-1 is likely to engage both local gut/portal–neural axes and systemic endocrine pathways, whose relative contributions are under investigation.

3. We assessed whether repeated oral administration of GIFT elicits immune responses, specifically by measuring IgG1 and IgG2c. We found no increases in IgG1 or IgG2c in mice treated with $GIFT_{Lux}$- or $GIFT_{GLP\text{-}1}$ (**Extended Data Fig. 10t, u**), suggesting that GIFT did not elicit a detectable adaptive humoral immune response

4. In the text of the revised manuscript, we now explicitly acknowledge several limitations of the study, including the use of oxygen-dependent reporters for visualization, relatively small cohort sizes, the limited interpretability of HbA1c changes due to the erythrocyte lifespan in mice, the absence of a concurrent chassis-only control in the monkey study, reliance on a plasmid-based genetic circuit, and the use of antibiotic resistance markers.

5. In the revised manuscript, we now include descriptions of dosing frequency and statements regarding the dose-dependent efficacy differences observed in both our mouse and monkey studies.

6. The Discussion section of the revised manuscript now addresses the potential contributions of local gut, hepatoportal, and neural GLP-1 signaling pathways to the therapeutic efficacy of GIFT.

7. The Discussion section of the revised manuscript now outlines multiple steps that will be required to advance the clinical translation of GIFT.

Please kindly note that we have revised the manuscript to comply with the journal's word-count requirements, as guided by the editor. All changes have been tracked, and revisions made in response to the reviewers' comments are highlighted in yellow.

Referee #1 (Remarks to the Author):

The authors have conducted an array of experiments that address the major concerns raised. Key points for further strengthening would be: inclusion of an intermediate-strength constitutive promoter or mention of how the results for this may compare, and description of efficacy differences between single and multiple doses (how often does the probiotic need to be administered?). The responses from comments should be explicitly discussed in the main text and/or that the reviewer responses be published, as they contain critical details necessary for a full evaluation of the study (*e.g.,* the promoter engineering advances, secretion details, and correct citations). Overall, the work is highly innovative in its application, precedence and efficacy established in complex models.

Reply:

We sincerely thank the referee for the positive and encouraging evaluation of our work, as well as for the constructive guidance that helped further strengthen the manuscript.

(1) Comparison with intermediate-strength constitutive promoters

Kindly recall from our response to comment #1 from the second round of review, we have experimentally evaluated moderate constitutive promoters (P_{lacUV5} and P_{lac}). While these promoters supported stable GLP-1 expression, the resulting secretion levels were insufficient to achieve meaningful glycemic control in db/db mice (**Supplementary Fig. 7**).

We acknowledge that, in principle, an intermediate-strength constitutive promoter could be further tuned to produce GLP-1 at concentrations sufficient to lower blood glucose. However, beyond achieving an appropriate expression level, an important consideration for GLP-1–based therapy is the temporal pattern of hormone exposure. Constitutive expression inherently leads to continuous GLP-1 production and persistent receptor activation, which may increase the risk of adverse effects and receptor desensitization over time (Kim et al., 2025; Baggio et al., 2004). In contrast, our glucose-responsive GIFT system produces GLP-1 only when glucose levels are elevated, thereby more closely matching physiological demand and avoiding prolonged, non-physiological receptor activation. We therefore view glucose-inducible regulation as both a means to achieve sufficient GLP-1 levels and as a strategy to improve long-term safety relative to constitutive expression. This conceptual distinction is now explicitly discussed in our revised Discussion. Please see **page 14** in the revised manuscript and below:

> "Although orally delivered engineered bacteria constitutively expressing GLP-1 have shown therapeutic potential in mice[40,41], sustained production could in theory limit achievable protein output, impose a metabolic burden on the microbial chassis[42], compromise protein stability, and/or increase the risk of prolonged, non-physiological receptor stimulation and desensitization[43]. In contrast, our glucose-responsive GIFT system produces GLP-1 only when glucose levels are elevated, thereby more closely matching physiological demand and avoiding prolonged, non-physiological receptor activation."

References:

Kim JA, Yoo HJ. Exploring the Side Effects of GLP-1 Receptor Agonist: To Ensure Its Optimal Positioning. Diabetes Metab J. 2025 Jul;49(4):525-541.

Baggio LL. et al. Chronic exposure to GLP-1R agonists promotes homologous GLP-1 receptor desensitization in vitro but does not attenuate GLP-1R-dependent glucose homeostasis in vivo. Diabetes. 2004 Dec;53 Suppl 3:S205-14.

(2) Single dosing versus repeated dosing

During the first round of peer review, we evaluated both single-dose and repeated oral administration of GIFT. In mice, a single dose elicited a transient glucose-lowering effect, with blood glucose levels returning to baseline within 24 hours—likely reflecting rapid intestinal transit and clearance of the engineered probiotic. In contrast, once-daily (repeated) dosing produced sustained glycemic control. Notably, in non-human primates, oral dosing every three days was sufficient to maintain therapeutic efficacy, consistent with slower gastrointestinal transit and reduced bacterial clearance compared with mice. Investigator-initiated clinical studies are currently being planned to further evaluate dosing frequency in humans. To clarify the administration regimen required to achieve and maintain therapeutic benefit, we have added a clear description of dosing frequency and the corresponding efficacy differences in the main text. Please see **page 7** and **page 13** in the revised manuscript and below:

> "Given that a single dose led to transient glucose lowering (with blood glucose returning to baseline within 24 hours, ostensibly owing to rapid clearance in the murine intestine), we conducted a 30-day experiment in which db/db mice were given a once-daily oral dose of 10^9 CFU $GIFT_{GLP\text{-}1}$."

> "A single oral dose of $GIFT_{GLP\text{-}1}$ (2×10^{11} CFU/kg) rapidly increased serum GLP-1 and plasma insulin levels (**Fig. 5b-e**), accompanied by a significant reduction in blood glucose that persisted for up to 3 days (**Fig. 5f**)."

(3) Happily, in line with *Nature*'s editorial policy, our point-by-point responses will be made publicly available upon acceptance of the manuscript. Having said that, we have endeavored to incorporate the reviewers' suggestions into the main text and Supplementary Information to the fullest extent permitted by journal's (quite limiting) length constraints.

Once again, we sincerely thank the referees for the thoughtful and constructive feedback, which substantially improved both the study and the manuscript.

Referee #2 (Remarks to the Author):

The authors have fully addressed my questions.

Reply:

Thanks for your positive feedback on our revised manuscript.

Referee #3 (Remarks to the Author):

Summary

This manuscript describes the engineering of an *E. coli* Nissle 1917 probiotic (termed GIFT) that senses intestinal glucose via a synthetic HexR-based promoter and secretes GLP-1 in a dose-dependent, reversible manner. The system achieves dynamic glycemic control in diabetic mice and non-human primates, with additional benefits on lipid metabolism, insulin sensitivity, and diabetic complications. The authors perform extensive safety profiling and benchmark the approach against semaglutide, a clinically approved GLP-1 analogue.

The work is technically rigorous, translationally relevant, and represents a significant step toward "smart" probiotic-based living medicines for metabolic disorders. The breadth of data (molecular, cellular, murine models, and NHPs) is impressive, and the concept of real-time, on-demand drug release addresses a critical unmet need in diabetes therapy.

Reply:

We sincerely thank the referee for the encouraging comments.

Major Comments

1. Pharmacokinetics and Mechanism of Action

- GLP-1 exposure from GIFT is markedly lower than semaglutide (40-250 PM vs 6000-12000 PM over 6h), yet efficacy is achieved. This suggests local gut-portal signaling may dominate.
- Recommendation: Include portal vein sampling or discuss explicitly how local versus systemic GLP-1 contributes to efficacy.

Reply:

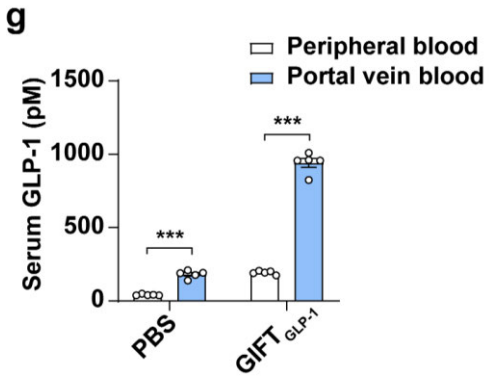
Thank you for this insightful comment. To directly address this point, we performed portal vein sampling following oral administration of GIFT and observed significantly higher GLP-1 levels in portal venous blood than in peripheral circulation (new **Fig. 2g**), indicating a strong portal-systemic gradient. Thus, GIFT-derived GLP-1 is likely to engage local gut-portal sensing and neural pathways, in addition to systemic endocrine signaling (Paternoster et al., 2018; Donath et al., 2013; Zhang et al., 2022; Holst et al., 2022; Beutler et al., 2026).

We recognize that a more detailed dissection of the relative contributions of local versus systemic GLP-1 will require additional targeted animal studies. However, given the short revision timeline as guided by the Journal editor (and thus a need to address the remaining comments primarily through existing data and text-based clarification), such experiments were not feasible within the current study. We have incorporated the new portal vein sampling results and an expanded discussion of local versus systemic GLP-1 signaling into the revised manuscript. Please see **page 7** and **page 15** in the revised manuscript and below:

"GLP-1 concentrations in portal vein blood 2 hours after administration were significantly higher than those measured in peripheral circulation (**Fig. 2g**), indicating a strong portal-systemic gradient, Thus, GIFT-derived GLP-1 is likely to engage local gut-portal sensing and neural pathways, in addition to systemic endocrine signaling[28]."

"We propose that GIFT-derived GLP-1 acts through a combination of systemic, intestinal,

hepatoportal, and neural pathways[44,45], the relative contributions of which remain to be defined and are under investigation."



Response Document Figure 1 (Fig. 2g in the revised manuscript). GLP-1 levels in portal vein blood and peripheral blood measured 2 hours after oral GIFT$_{GLP-1}$ administration. Data are expressed as means ± SEM; *n* = 5 mice. *P*-values were calculated using two-way ANOVA followed by Tukey's post hoc multiple-comparison test. ***$P < 0.001$.

References:

Paternoster S, Falasca M. Dissecting the Physiology and Pathophysiology of Glucagon-Like Peptide-1. Front Endocrinol (Lausanne). 2018.9:584.

Donath MY, Burcelin R. GLP-1 effects on islets: hormonal, neuronal, or paracrine? Diabetes Care. 2013.36 Suppl 2(Suppl 2):S145-8.

Zhang T, Perkins MH, Chang H, Han W, de Araujo IE. An inter-organ neural circuit for appetite suppression. Cell. 2022.185(14):2478-2494.e28.

Holst JJ, Andersen DB, Grunddal KV. Actions of glucagon-like peptide-1 receptor ligands in the gut. Br J Pharmacol. 2022.179(4):727-742.

Beutler LR. GLP-1 physiology and pharmacology along the gut-brain axis. J Clin Invest. 2026.136(2):e194744.

2. Microbiome and Ecological Impact

- Colonization is transient, but long-term repeated dosing could alter the gut microbiome.
- Recommendation: Provide 16S rRNA profiling or at minimum discuss microbiome modulation as both a risk and potential therapeutic synergy.

Reply:

Thank you for this constructive guidance. During the peer review period, we performed 16S rRNA gene sequencing to assess whether repeated oral dosing of GIFT$_{GLP-1}$ alters the gut microbiome in db/db mice.

Relative to PBS controls, GIFT$_{GLP-1}$ treatment was associated with a significant increase in *Faecalibacterium prausnitzii* and significant decreases in *Enterococcus faecalis* and *Acetatifactor*

muris (new **Supplementary Fig. 8**). These shifts are directionally consistent with prior literature linking *F. prausnitzii* to anti-inflammatory activity and improved metabolic phenotypes (Miquel et al., 2013; Xu et al., 2020; Maioli et al., 2021), whereas *E. faecalis* and its virulence factors have been implicated in inflammation and tissue injury in mouse models (Duan et al., 2019; Quaglio et al., 2021). *A. muris* has been reported as an inflammation-associated taxon enriched under high-fat diet/obesity contexts (Lee et al., 2019; Just et al., 2018). In addition, linear discriminant analysis (LDA) identified *Clostridia*, *Roseburia*, *Ruminococcaceae*, and *Clostridium cluster* IV as the most informative taxa distinguishing the $GIFT_{GLP\text{-}1}$ group from the PBS/$GIFT_{Lux}$ groups (new **Supplementary Fig. 9**); these taxa have been reported to show negative associations with type 2 diabetes and related metabolic dysfunction (Gurung et al., 2020; Grenda et al., 2022; Yan et al., 2021; Yan et al., 2023).

Together, these data indicate that repeated $GIFT_{GLP\text{-}1}$ administration can modulate the gut microbiome. The new data have been incorporated into the main text (**page 9**) as follows:

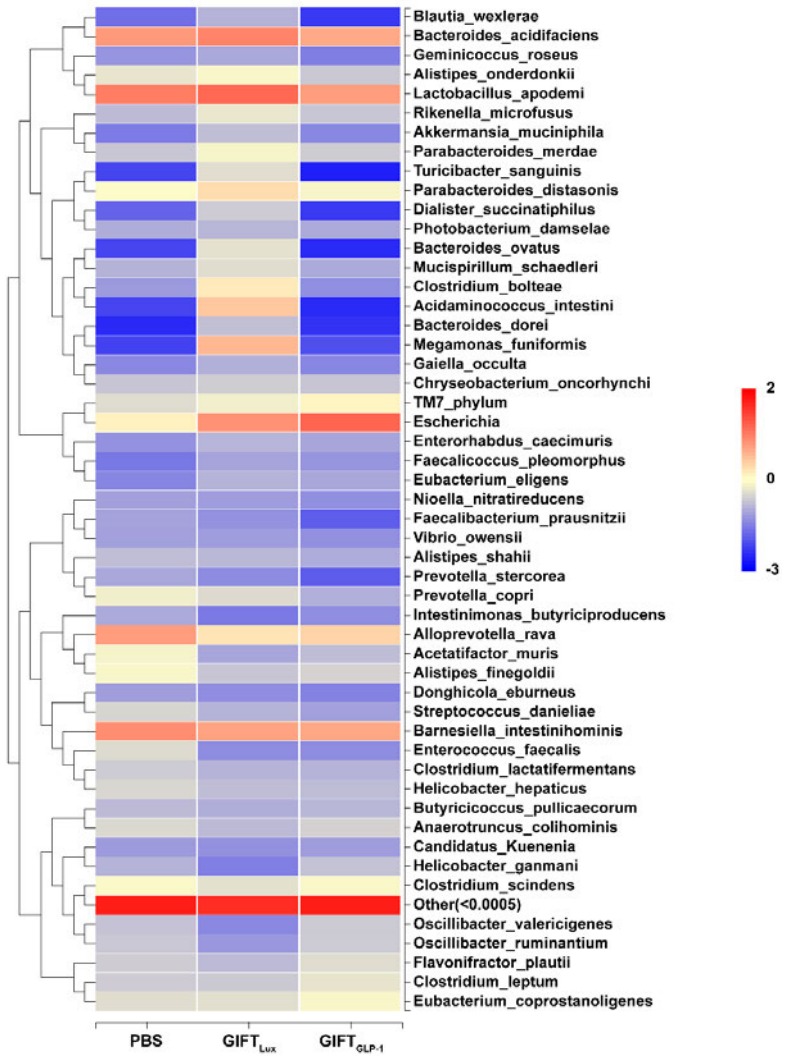
"Finally, 16S rRNA sequencing revealed that $GIFT_{GLP\text{-}1}$ modulated the gut microbiome, increasing the relative abundance of the beneficial commensal *Faecalibacterium prausnitzii* while reducing potentially pathogenic and inflammation-associated taxa, including *Enterococcus faecalis* and *Acetatifactor muris* compared with PBS controls[33-35] (**Supplementary Fig. 8**). A linear discriminant analysis (LDA) identified *Clostridia*, *Roseburia*, *Ruminococcacease*, and *Clostridium* IV as key taxa discriminating the $GIFT_{GLP\text{-}1}$ group from PBS/$GIFT_{Lux}$-administered groups (**Supplementary Fig. 9**)."

Moreover, we now explicitly discuss this modulation in the manuscript in two ways: (i) as a safety consideration for long-term, repeated dosing (*i.e.*, the need to monitor microbiome changes), and (ii) as a potential therapeutic synergy, given the established role of microbiome composition in metabolic homeostasis. Pleased see **page 15** in the revised manuscript and below:

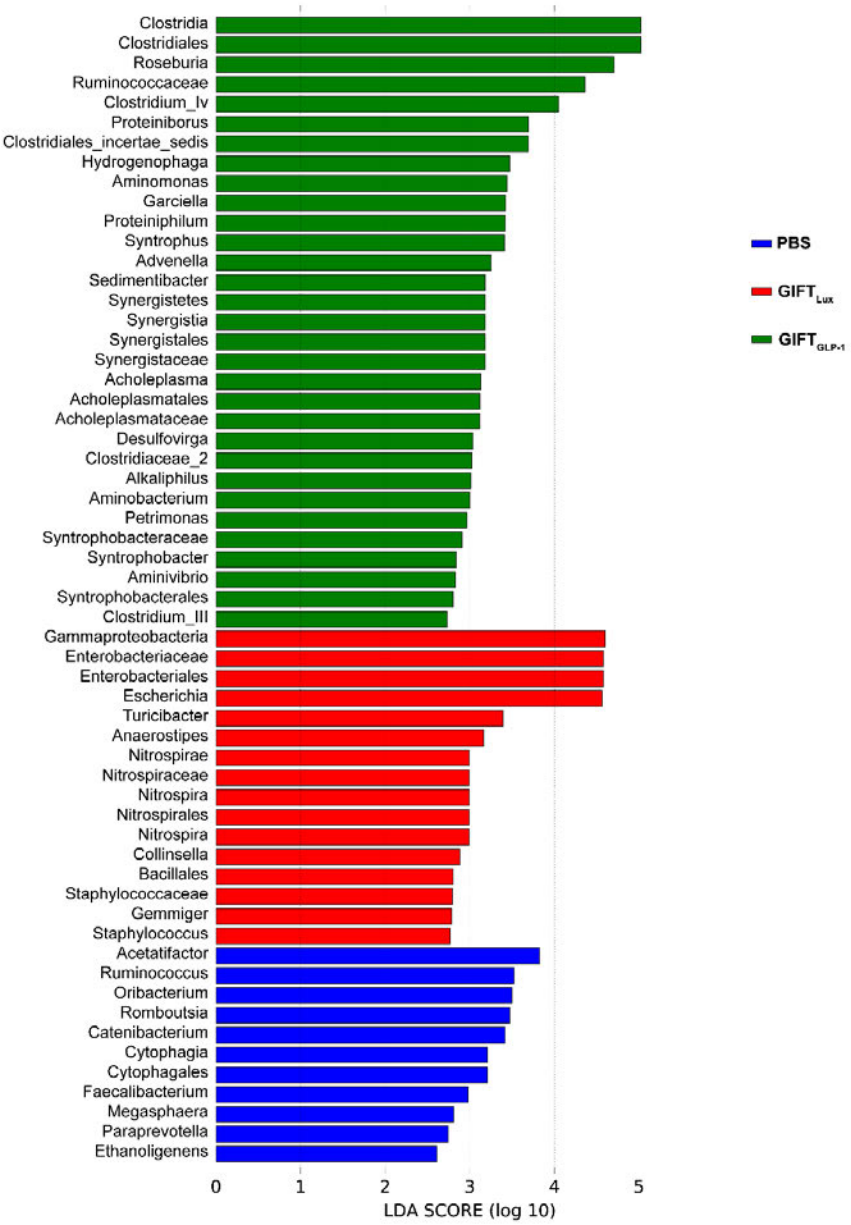
> "Notably, repeated $GIFT_{GLP\text{-}1}$ administration was associated with measurable modulation of the gut microbiota, which should be monitored as a safety consideration for long-term dosing, while also potentially contributing to therapeutic efficacy given the established role of the gut microbiome in metabolic homeostasis[48]."

However, kindly noting that our gut microbiome analysis in mice was conducted with a sample size of five animals per group. This represents a limitation, given that gut microbial communities are characterized by high inter-individual heterogeneity (Miller et al., 2021). Consequently, a small cohort size severely compromises the statistical power to distinguish $GIFT_{GLP\text{-}1}$-associated shifts in microbial composition from baseline biological variability. Therefore, the current microbiome findings should be considered preliminary and require further validation in larger cohorts to enhance their robustness and generalizability. This limitation has been acknowledged in the Discussion section. Pleased see **page 16** in the revised manuscript and below:

> "Some metabolic endpoints were assessed in small cohorts. In particular, the gut microbiome profiling in mice was performed with five animals per group, which may not fully capture inter-individual variation within the microbial community and requires further validation in expanded cohorts."



Response Document Figure 2 (Supplementary Fig. 8 in the revised manuscript). Gut microbiota composition in db/db mice undergoing different treatments. db/db mice were administered PBS, GIFT$_{Lux}$, or GIFT$_{GLP-1}$ daily for 30 days. Fecal samples were collected 6 days after the final gavage for microbiome analysis. Taxonomic profiles are shown at the species level. Species with a relative abundance ≥0.5% in at least one sample are displayed individually, whereas species with lower abundance were grouped by phylum and labeled as "Others". Heatmap colors represent Z-score–normalized, log-transformed relative abundances of each species across samples, with values ranging from −3 to 2, indicating the deviation (in standard deviations) of a species' abundance in a given sample from its mean abundance across all samples. Hierarchical clustering along the vertical axis reflects similarities in species-level microbial community composition among samples.



Response Document Figure 3 (Supplementary Fig. 9 in the revised manuscript). Linear discriminant analysis effect size (LEfSe) difference analysis among db/db mice undergoing different treatments. db/db mice were treated with PBS, $GIFT_{Lux}$, or $GIFT_{GLP\text{-}1}$ daily for 30 days. Fecal samples were collected 6 days after the final gavage and subjected to microbiome analysis. Bars represent the linear discriminant analysis (LDA) scores (log_{10}), indicating the effect size of each taxon contributing to differences among treatment groups. Only taxa with an LDA score ≥2 were considered discriminative and are shown.

References:

Miquel, S. et al. *Faecalibacterium prausnitzii* and human intestinal health. Current Opinion In Microbiology. 2013. 16, 255-261.

Xu, J. et al. Faecalibacterium prausnitzii-derived microbial anti-inflammatory molecule regulates intestinal integrity in diabetes mellitus mice via modulating tight junction protein expression. Journal of Diabetes. 2020. 12, 224-236.

Maioli, T. U. et al. Possible Benefits of *Faecalibacterium prausnitzii* for Obesity-Associated Gut Disorders. Frontiers in Pharmacology. 2021. 12, 740636.

Quaglio, A. E. V., Grillo, T. G., De Oliveira, E. C. S., Di Stasi, L. C. & Sassaki, L. Y. Gut microbiota, inflammatory bowel disease and colorectal cancer. World Journal of Gastroenterology. 2022. 28, 4053-4060.3.

Duan, Y. et al. Bacteriophage targeting of gut bacterium attenuates alcoholic liver disease. Nature. 2019. 575, 505-511.

Lee, C. et al. CD1d modulates colonic inflammation in $NOD^{2-/-}$ mice by altering the intestinal microbial composition comprising *Acetatifactor muris*. J Crohns Colitis. 2019.13, 1081-1091.

Just, S. et al. The gut microbiota drives the impact of bile acids and fat source in diet on mouse metabolism. Microbiome. 2018. 6, 1-18.

Gurung, M. et al. Role of gut microbiota in type 2 diabetes pathophysiology. EBioMedicine. 2020. 51, 102590.

Grenda, T. et al. Probiotic potential of *Clostridium* spp.-advantages and doubts. Current issues in molecular biology. 2022. 44, 3118-3130.

Yan, J. K. et al. Structural characteristics and ameliorative effect of a polysaccharide from Corbicula fluminea industrial distillate against acute liver injury induced by CCl(4) in mice. International Journal of Biological Macromolecules. 2023. 227, 391-404.

Yan, X. et al. Construction of a sustainable 3-hydroxybutyrate-producing probiotic *Escherichia coli* for treatment of colitis. Cell Mol Immunol. 2021. 18, 2344-2357.

3. Variability in Glucose Thresholds

- The activation threshold (~10–11 mM) is clinically relevant, but inter-individual differences in postprandial glucose kinetics may affect performance.
- Recommendation: Consider intermediate hyperglycemia models (*e.g.*, diet-induced prediabetes) to demonstrate robustness of the sensor.

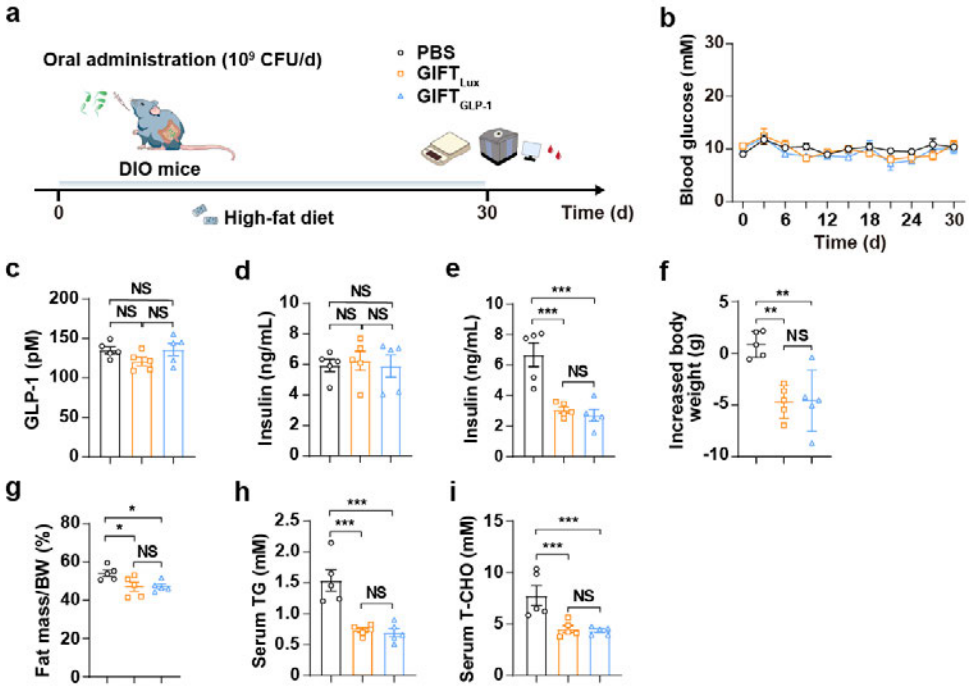
Reply:

Thank you for this insightful comment. During the first round of peer review, we evaluated GIFT performance in a high-fat diet (HFD)-induced obese (DIO) mouse model, which exhibits mild/intermittent dysglycemia resembling a prediabetic state (O'Brien et al., 2018; Winzell. et al., 2004).

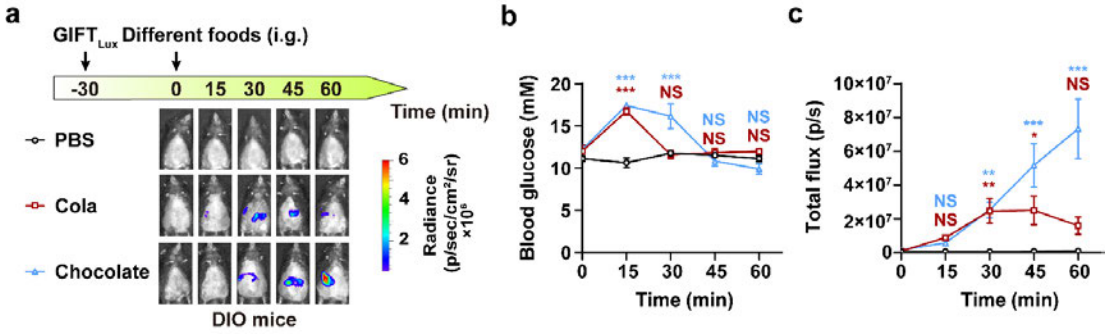
DIO mice received a daily oral dose of 10^9 CFU of $GIFT_{GLP\text{-}1}$ or the control strain ($GIFT_{Lux}$), and fasting blood glucose levels were monitored every three days for 30 days. In this model, neither $GIFT_{Lux}$ nor $GIFT_{GLP\text{-}1}$ significantly altered blood glucose levels, as GLP-1 and insulin levels were not induced (**Extended Data Fig. 9**), consistent with the minimal glucose elevations in DIO mice being below the activation threshold of the GIFT sensor.

We next examined whether postprandial glucose spikes—more variable across individuals and clinically relevant in early metabolic disease— could activate GIFT in this model. Intragastric administration of high-sugar foods such as cola (11.2 g carbohydrates per 100 mL, 10 μL/g) or melted chocolate (43 g carbohydrates per 100 g, 10 μL/g) induced LuxCDABE expression in GIFT cells (**Fig. 3a-c** and **Supplementary Fig. 7**). Importantly, when DIO mice dosed with $GIFT_{GLP\text{-}1}$ (10^9 CFU) consumed cola or chocolate, the magnitude of the postprandial glucose spikes was significantly reduced compared with $GIFT_{Lux}$-treated controls (**Fig. 3g-i**), indicating that postprandial glucose increases after high-sugar intake are sufficient to activate GIFT, thereby blunting postprandial hyperglycemia.

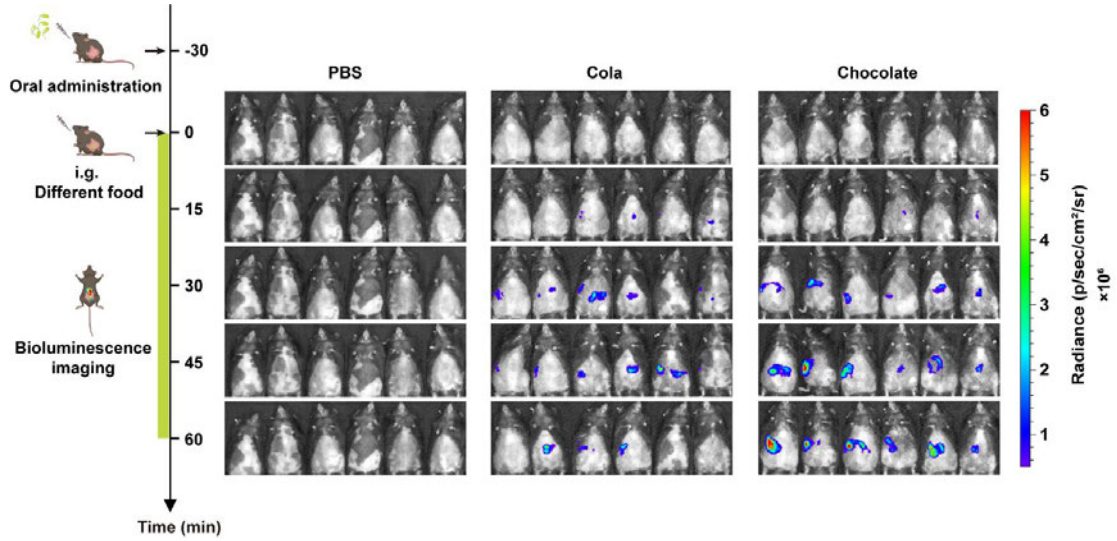
Collectively, these results show that GIFT exhibits a desirable "quiet-at-baseline, responsive-on-demand" behavior: it remains largely inactive under modest fasting glycemia yet responds dynamically to postprandial glucose spikes. This demonstrates that the sensor effectively responds across physiologically relevant glucose ranges and suggests a potential preventive/early-intervention use case in prediabetes, particularly for mitigating postprandial glucose spikes following intake of high-sugar foods and beverages.



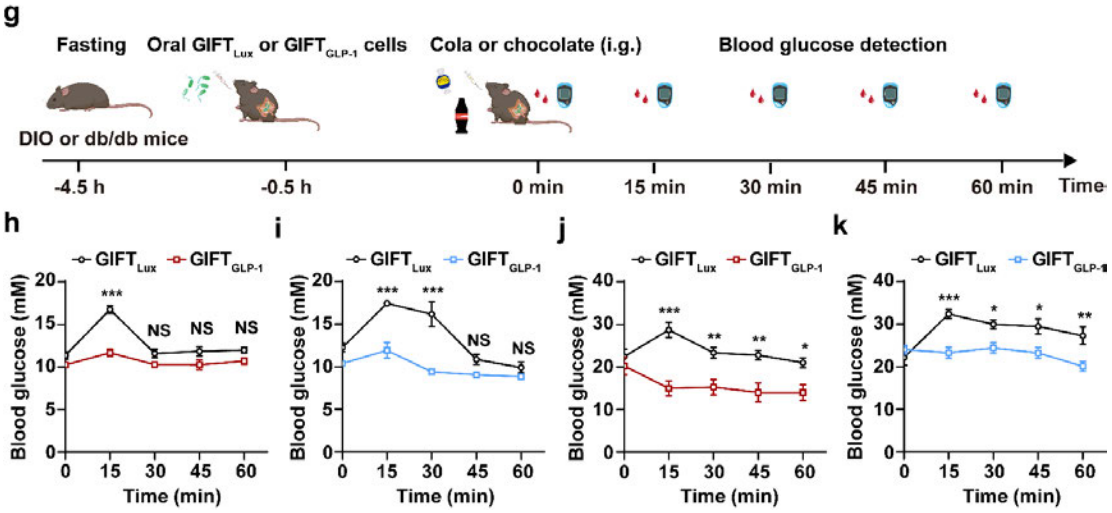
Response Document Figure 4 (Extended Data Fig. 9 in the revised manuscript): GIFT-mediated diabetes and obesity management in DIO mice. a, Schematic timeline for GIFT-mediated long-term glycemic control in DIO mice. Mice were treated with PBS, $GIFT_{Lux}$, or $GIFT_{GLP-1}$ cells (10^9 CFU) daily for 30 days. Serum GLP-1 and plasma insulin were measured 2 hours after the first gavage, and fasting blood glucose was monitored every three days. Lipid profiles and plasma insulin were evaluated 24 hours after the final gavage. **b-i,** Data for DIO mice: fasting blood glucose (**b**), serum GLP-1 (**c**), plasma insulin levels 2 hours after the first gavage (**d**) and 24 hours after the final gavage (**e**), body weight (**f**), fat mass (**g**), serum TG (**h**), and T-CHO levels (**i**). Data in **b-i** are expressed as means ± SEM; $n = 5$ mice. *P*-values were calculated by one-way ANOVA. NS, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.



Response Document Figure 5 (Fig. 3a-c in the revised manuscript): Effects of oral administration of different sugary foods on blood glucose levels and GIFT-mediated gene expression dynamics in DIO mice. Mice were orally administered $GIFT_{Lux}$ cells (10^9 CFU). 30 min later, they received intragastric (i.g.) administration of PBS, cola, or chocolate. Blood glucose levels (**b**) and bioluminescence signal intensity (**a**, **c**) were monitored every 15 minutes. Data in panels **b** and **c** are expressed as means ± SEM; $n = 6$ mice. P-values were calculated using two-way ANOVA followed by Tukey's post hoc multiple-comparison test. NS, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.



Response Document Figure 6 (Supplementary Fig. 7 in the revised manuscript): Effects of different sugary foods on GIFT-mediated gene expression dynamics in DIO mice. DIO mice were orally administered $GIFT_{Lux}$ cells (10^9 CFU). Thirty minutes post-gavage, the mice were administered intragastrically (i.g.) with PBS, cola, or chocolate. Imaging was performed every 15 min using an *in vivo* imaging system.



Response Document Figure 7 (Fig. 3g-i in the revised manuscript): GIFT-mediated postprandial glycemic control in high-fat diet-induced obese (DIO) mice. g, Time schedule for oral administration of $GIFT_{GLP-1}$ cells for postprandial glycemic control in DIO and db/db mice. After a 4-hour fasting period, mice were orally administered either $GIFT_{Lux}$ or $GIFT_{GLP-1}$ cells (10^9 CFU) and received cola or chocolate 30 min later. Blood glucose levels were monitored every 15 minutes. **h**, **i**, Blood glucose levels in DIO mice following intragastric administration of cola (**h**) or chocolate (**i**). Data in panels **h-i** are expressed as means ± SEM; $n = 6$ mice. P-values were calculated using two-

way ANOVA followed by Tukey's post hoc multiple-comparison test. NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001.

References:

O'Brien PD. et al., Juvenile murine models of prediabetes and type 2 diabetes develop neuropathy. Dis Model Mech. 2018.11(12):dmm037374.

Winzell MS, Ahrén B. The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. Diabetes. 2004.53 Suppl 3:S215-9.

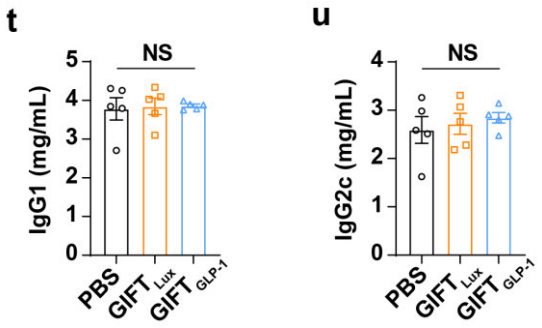
4. Adaptive Immunogenicity

- The safety analysis is comprehensive for acute responses, but adaptive immunity is not addressed.
- Recommendation: Assess antibody responses to GLP-1 or bacterial components in long-term studies.

Reply:

Thank you for this constructive guidance. We assessed immune responses using archived serum samples collected during our previous long-term dosing studies. Specifically, we measured IgG1 and IgG2c, which are commonly used to evaluate Th2- and Th1-based adaptive immune responses in mice. No significant increases in IgG1 or IgG2c levels were observed in GIFT$_{Lux}$- or GIFT$_{GLP\text{-}1}$-treated mice as compared with PBS control, suggesting that repeated oral administration did not elicit a detectable adaptive immune response under the conditions tested. These findings have been incorporated into the main text (**Extended Data Fig. 10t, u**). Please see **page 13** in the revised manuscript and below:

"Moreover, repeated oral administration of GIFT$_{GLP\text{-}1}$ did not elicit a detectable adaptive immune response (**Extended Data Fig. 10t,u**), supporting the favorable safety profile of our gut-resident, orally delivered therapeutic system. "



Response Document Figure 8 (Extended Data Fig. 10t, u in the revised manuscript): Serum levels of IgG1 (**t**) and IgG2c (**u**) in db/db mice after one-month treatment with PBS, GIFT$_{Lux}$ (10^9 CFU/day), or GIFT$_{GLP\text{-}1}$ (10^9 CFU/day). Data in **t-u** are expressed as means ± SEM; n = 5 mice. P-values were calculated using one-way ANOVA followed by Tukey's post hoc multiple-comparison test. NS, not significant.

5. Statistical Rigor and Sample Size

- Some critical endpoints (like HbA1c) rely on small cohorts (n=5).
- Recommendation: Provide power calculations or acknowledge as a limitation.

Reply:

Thank you for this constructive guidance. We recognize that certain endpoints, including HbA1c measurements, were evaluated in small cohorts, and understand that the statistical power is limited for detecting subtle effects. We now explicitly state this limitation in the revised manuscript. Please see **page 15** in the revised manuscript and below:

> "Some metabolic endpoints were assessed in small cohorts, and HbA1c changes in mice should be interpreted cautiously, as the 30-day treatment period does not span a full erythrocyte lifespan (~40-45 days)[49]."

Minor Comments

• Clarify whether the final GIFT construct is plasmid-borne or chromosomally integrated. Stability will influence translational feasibility.

Reply:

Thank you for raising this important point. The current GIFT construct is plasmid-borne rather than chromosomally integrated. We adopted this architecture to facilitate rapid prototyping and modular optimization of the sensing and secretion modules, as well as flexible tuning of expression levels during development. However, we recognize that plasmid-based expression can pose limitations for long-term genetic stability and therefore affects translational feasibility. Accordingly, we have now explicitly stated in the revised Discussion one step towards clinical translation will involve generating a more stable and biosafe genetic configuration (likely chromosomal integration). Please see **page 16** in the revised manuscript and below:

> "The current plasmid-based circuit was useful for facilitating rapid prototyping; however, the prospects for the clinical translation of GIFT would be improved by generating cells with stable chromosomal integration of the circuit, as well as removal of antibiotic resistance markers."

• Rephrase Line 83-84 ("no research has been conducted…") to acknowledge prior probiotic engineering for metabolic regulation in animal model, while emphasizing novelty in glucose-responsive GLP-1 release in NHP and mice.

Reply:

Thank you for this constructive guidance. We recognize that prior studies have engineered probiotic/bacterial systems for metabolic regulation in animal models, including constitutive GLP-1 expression, and we have revised the text to acknowledge these literatures while clarifying our specific advance in both Introduction and Discussion. Please see **page 2** and **page 14** in the revised manuscript and below:

> "Engineered probiotic-based living drugs have been developed for metabolic regulation by consuming dietary or gut-derived toxic metabolites[16,17] or secreting therapeutic agents[18]."

> "Although orally delivered engineered bacteria constitutively expressing GLP-1 have shown therapeutic potential in mice[40,41], sustained production could in theory limit achievable protein output, impose a metabolic burden on the microbial chassis[42], compromise protein stability,

and/or increase the risk of prolonged, non-physiological receptor stimulation and desensitization[43].”

• Consider expanding the Discussion with regulatory considerations for engineered probiotics (e.g., GMP production, GMO classification).

Reply:

Thank you for this valuable guidance. We have now expanded the Discussion to explicitly address regulatory considerations, including development of clinically compatible formulations, GMP-compliant manufacturing and quality control, biosafety and genetic stability assessments, and regulatory classification/oversight as genetically modified microorganisms (GMMs)/GMOs. Please see **page 16** in the revised manuscript and below:

“Ongoing efforts to develop clinically compatible formulations, together with good manufacturing practice (GMP) manufacturing, biosafety and stability assessments, and clear regulatory classification as genetically modified microorganisms, will facilitate the clinical translation of GIFT.”

Notably, the U.S. FDA has already approved several engineered probiotic products (https://zbiotics.com/?srsltid=AfmBOoqDUa03EK-VONe2PUvCj6ZR-Ewcj_VFU1k_u-AJtQ8IwNdvrMYN) under established regulatory frameworks (for example, GRAS pathways for food and dietary applications), highlighting the feasibility of advancing genetically modified probiotics toward human use. These precedents increase confidence that GIFT, with appropriate safety and manufacturing controls, can be realistically developed as a therapeutic modality for diabetes.

Strengths

• Clear innovation: first oral probiotic platform enabling real-time, glucose-responsive GLP-1 release.

• Streamlined engineering of a HexR-based sensor with clinically aligned activation thresholds.

• Comprehensive validation across multiple murine models and spontaneous diabetic cynomolgus monkeys.

• Strong translational framing, including direct comparison to semaglutide.

• Extensive acute and subchronic safety profiling.

Recommendation

This is a great manuscript with novelty and broad interest, well-suited for a high-impact journal like Nature. I recommend acceptance after major revisions, addressing the mechanistic, ecological, and immunogenicity concerns outlined above.

We express our sincere gratitude for the helpful guidance in improving our study and manuscript. Many thanks!

Referee #4 (Remarks to the Author):

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

Reply:

We sincerely thank the referee for the encouraging comments and the valuable guidance, which have helped us strengthen both the study and the manuscript.

Referee #5 (Remarks to the Author):

This manuscript describes the development of GIFT (Glucose-Responsive Intestinal Functional Therapeutics), an engineered probiotic system using *E. coli* Nissle 1917 as a chassis for glucose-responsive delivery of GLP-1. The core innovation is a synthetic glucose-sensing circuit based on the HexR transcriptional repressor from Neisseria meningitidis, which is derepressed by the glycolytic intermediate KDPG when glucose levels exceed approximately 10 mM. The authors demonstrate that this system activates gene expression preferentially in diabetic mice with elevated blood glucose while remaining quiescent in normoglycemic animals. When engineered to produce a secreted GLP-1 variant, the system reduces fasting blood glucose, improves glucose tolerance, lowers HbA1c, and ameliorates several diabetic complications in db/db mice over 30-day treatment periods. The authors extend these findings across multiple mouse models (DIO, DIO+STZ) showing that efficacy correlates with disease severity, and provide supporting data from a small non-human primate study. The work is positioned as proof-of-concept for a "microbial incretin mimic" approach to chronic glycemic management rather than real-time closed-loop glucose control.

The manuscript presents an ambitious and technically sophisticated body of work that addresses a genuine unmet need in diabetes therapeutics. The fundamental concept of glucose-responsive therapeutic delivery from an orally administered probiotic is scientifically compelling, and the authors have assembled a substantial dataset spanning in vitro characterization, multiple rodent disease models, and preliminary primate studies.

Several aspects of the work are commendable: the demonstration of differential activation between normoglycemic and hyperglycemic mice provides direct functional evidence of glucose-responsiveness in vivo; the $GIFT_{Lux}$ control appropriately isolates GLP-1-dependent effects from chassis effects in the primary efficacy experiments; and the multi-model validation adds confidence that observations are not model-specific artifacts. The comparison to semaglutide safety profiles raises interesting questions about the potential advantages of physiological-level versus supraphysiological GLP-1 delivery. The comprehensive safety data, including the two-month comparative study with semaglutide examining complete blood counts, serum biochemistry, and inflammatory markers, substantially strengthens the translational case. The body composition data suggesting potential preservation of lean mass with $GIFT_{GLP-1}$ treatment compared to semaglutide is intriguing and could represent a meaningful clinical advantage if confirmed in further studies.

The characterization of the secretion mechanism, including SecA inhibitor experiments and quantification of secretion efficiency, is thorough. The demonstration that GLP-1 released through bacterial lysis lacks biological activity due to retention of the uncleaved signal peptide effectively addresses the question of whether active secretion versus passive lysis drives therapeutic effects. The comparison with constitutive expression systems (both strong and moderate promoters) provides valuable context for why glucose-responsive regulation is advantageous.

However, several issues require attention. The manuscript does not address the colibactin genotoxicity concern associated with the pks genomic island present in *E. coli* Nissle 1917—a significant omission for a therapeutic intended for chronic daily administration. Some methodological details require clarification. The NHP data, while including appropriate healthy and untreated diabetic controls, lack a chassis-only control that would definitively isolate GLP-1-specific effects in primates. Furthermore, the mechanistic argument that portal vein delivery explains therapeutic efficacy at GLP-1 concentrations roughly two orders of magnitude below those achieved by approved GLP-1 agonists, while pharmacologically plausible, remains incompletely substantiated. The points below detail these and other concerns that should be addressed.

Reply:

Thank you for the positive and encouraging evaluation of our work and for raising several important points. We recognize that the colibactin-associated genotoxicity concern linked to the *pks* genomic island in *E. coli* Nissle 1917 is a relevant consideration for therapeutic strategies involving chronic or repeated administration. We also acknowledge the reviewer's concerns regarding the absence of a chassis-only control in the non-human primate study, as well as the need for a clearer mechanistic framework to explain the mode of action of GIFT. However, given the short revision timeline as guided by the Journal editor (and thus a need to address the remaining comments primarily through existing data and text-based clarification), additional *in vivo* experiments were not feasible within the current study. Accordingly, we have substantially revised the Discussion to explicitly address these issues.

Specific Concerns

The manuscript does not address the colibactin genotoxicity concern associated with the *pks* genomic island present in *E. coli* Nissle 1917. Multiple studies have demonstrated that EcN produces the genotoxin colibactin, which induces DNA double-strand breaks in epithelial cells and has been linked to colorectal cancer risk in mechanistic studies. While standard Ames tests may not detect this specific genotoxic mechanism, and while EcN has an extensive history of human use as a probiotic, a manuscript proposing chronic daily therapeutic administration of this chassis should at minimum discuss this controversy.

Reply:

Thank you for raising this important concern. We recognize that, despite the long history *E. coli* Nissle 1917 (EcN) use as a probiotic, the reported genotoxicity associated with colibactin production from the *pks* genomic island warrants explicit discussion—particularly for any scenario involving chronic or repeated therapeutic dosing. We also acknowledge that standard bacterial mutagenicity assays (*e.g.*, Ames) may not fully capture colibactin's host-cell DNA damage mechanism, underscoring the need for chassis-specific risk assessment beyond conventional genotoxicity screens.

To address this directly, we have revised the Discussion to (i) acknowledge the ongoing controversy and mechanistic evidence linking *pks* to epithelial DNA damage, and (ii) outline a clear mitigation path for translation. Specifically, we state that future clinically oriented versions of GIFT will incorporate established biosafety engineering, including deletion of the *pks* genomic island to eliminate colibactin biosynthesis. In addition, we note that the glucose-responsive circuit is modular and could be ported to alternative non-*E. coli* probiotic chassis (*e.g.*, lactic acid bacteria), although such chassis changes would require dedicated optimization of sensing and secretion modules. These points have been added to the revised Discussion. Please see **page 16** in the revised manuscript and below:

> "Given prior reports of colibactin-associated genotoxicity linked to the *pks* genomic island in EcN[50], future translational development will incorporate deletion of the *pks* island to enhance biosafety. Notably, the glucose-responsive gene circuit is modular and could, in principle, be ported to alternative probiotic chassis with favorable safety profiles, although such chassis substitution would require dedicated optimization of sensing and secretion modules."

The non-human primate study includes appropriate comparisons with healthy non-diabetic monkeys and untreated diabetic monkeys, which provides meaningful context for interpreting the magnitude of therapeutic effects. However, the study lacks a concurrent $GIFT_{Lux}$ (or other chassis-only) control group. While the within-subject pre-treatment baseline and the untreated diabetic control group

provide substantial protection against confounding, and while the mouse data with GIFT$_{Lux}$ controls strongly suggests effects are GLP-1-mediated, a parallel chassis-only control in primates would more definitively rule out that observed effects derive from chassis-related factors rather than GLP-1 specifically. Given the comprehensive GIFT$_{Lux}$ control data in rodents, this concern is somewhat mitigated, but explicit acknowledgment of this limitation would strengthen the manuscript.

Reply:

Thank you for this valuable guidance. We recognize that inclusion of a concurrent chassis-only control (*e.g.*, GIFT$_{Lux}$) in the non-human primate (NHP) study would enable definitive exclusion of chassis-related contributions and would further strengthen causal attribution to glucose-responsive GLP-1 release. Accordingly, we have now explicitly acknowledged this limitation in the Discussion. Please see **page 15** in the revised manuscript and below:

> "The monkey study lacked a chassis-only control, although mouse data and within-subject baselines support a GLP-1-mediated mechanism."

The GLP-1 ELISA methodology requires clarification regarding sample collection procedures. While the methods specify use of an active GLP-1 kit (Mercodia EGLP-35K), they do not indicate whether blood samples were collected with DPP-IV inhibitors, which is standard practice given the extremely short half-life of active GLP-1. If inhibitors were used, this should be stated; if not, the measured values may underestimate true active GLP-1 concentrations at the time of collection, which affects interpretation of the therapeutic mechanism.

Reply:

Thank you for this valuable guidance. The GLP-1 expressed by GIFT is a DPP-IV–resistant mutant, which has an extended half-life relative to wild-type GLP-1. Nevertheless, seeking to ensure accurate measurement of active GLP-1 and to prevent any residual degradation, note that indeed, all blood samples were collected in the presence of a DPP-IV inhibitor (486460-32-6, MedChemExpress). This procedure is now explicitly described in the revised Methods section. Please see **page 27** in the revised manuscript and below:

> "For serum GLP-1 measurements, blood samples were collected into tubes pre-coated with the DPP-IV inhibitor Sitagliptin (MK-0431, MedChemExpress)."

The manuscript claims therapeutic efficacy at systemic GLP-1 concentrations approximately 25-300 fold lower than those achieved by semaglutide. The proposed explanation—that intestinal delivery provides favorable portal vein pharmacokinetics mimicking native incretin secretion—is physiologically reasonable but not directly demonstrated. The temporal correlation between GLP-1 elevation and insulin secretion, combined with the GIFT$_{Lux}$ control data, provides circumstantial evidence that effects are GLP-1-mediated, but more direct mechanistic evidence would strengthen this claim.

Reply:

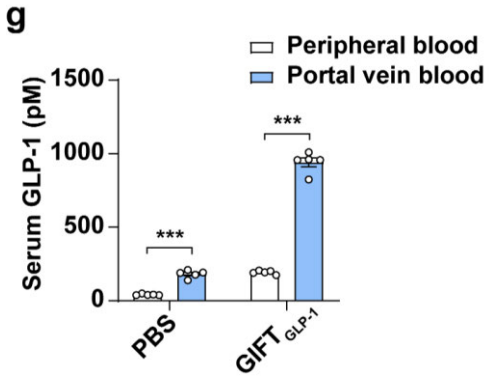
Thank you for this valuable guidance. We have now performed portal vein sampling following oral administration of GIFT and observed significantly higher GLP-1 levels in portal venous blood than those measured in peripheral circulation (new **Fig. 2g**), indicating a strong portal-systemic gradient. Thus, GIFT-derived GLP-1 is likely to engage local gut-portal sensing and neural pathways, in addition to systemic endocrine signaling (Paternoster et al., 2018; Donath et al., 2013; Zhang et al.,

2022). We thus propose that, in addition to its endocrine action after entering the circulation to potentiate glucose-stimulated insulin secretion, GLP-1 produced by GIFT can exert extra-pancreatic effects by activating GLP-1 receptors in the gut, liver, and nervous system. Locally generated GLP-1 engages intestinal GLP-1R–expressing cells and vagal afferent pathways to initiate gut-brain signaling, which modulates autonomic outputs and hepatic glucose metabolism (Holst et al., 2022). Through coordinated gut-brain-liver communication, these pathways complement the insulinotropic effects of circulating GLP-1 to reinforce and stabilize systemic glucose homeostasis (Beutler et al., 2026).

We recognize that a more detailed dissection of the relative contributions of local versus systemic GLP-1 will require additional targeted animal studies. However, given the short revision timeline as guided by the Journal editor (and thus a need to address the remaining comments primarily through existing data and text-based clarification), such experiments were not feasible within the current study. We have incorporated the new portal vein sampling results and an expanded discussion of local versus systemic GLP-1 signaling into the revised manuscript. Please see **page 7** and **page 15** in the revised manuscript and below:

> "GLP-1 concentrations in portal vein blood 2 hours after administration were significantly higher than those measured in peripheral circulation (**Fig. 2g**), indicating a strong portal-systemic gradient, Thus, GIFT-derived GLP-1 is likely to engage local gut-portal sensing and neural pathways, in addition to systemic endocrine signaling[28]."

> "We propose that GIFT-derived GLP-1 acts through a combination of systemic, intestinal, hepatoportal, and neural pathways[44,45], the relative contributions of which remain to be defined and are under investigation."



Response Document Figure 9 (Fig. 2g in the revised manuscript). GLP-1 levels in portal vein blood and peripheral blood measured 2 hours after oral GIFT$_{GLP-1}$ administration. Data are expressed as means ± SEM; n = 5 mice. P-values were calculated using two-way ANOVA followed by Tukey's post hoc multiple-comparison test. ***$P < 0.001$.

References:

Paternoster S, Falasca M. Dissecting the Physiology and Pathophysiology of Glucagon-Like Peptide-1. Front Endocrinol (Lausanne). 2018.9:584.

Donath MY, Burcelin R. GLP-1 effects on islets: hormonal, neuronal, or paracrine? Diabetes Care. 2013.36 Suppl 2(Suppl 2):S145-8.

Zhang T, Perkins MH, Chang H, Han W, de Araujo IE. An inter-organ neural circuit for appetite suppression. Cell. 2022.185(14):2478-2494.e28.
Holst JJ, Andersen DB, Grunddal KV. Actions of glucagon-like peptide-1 receptor ligands in the gut. Br J Pharmacol. 2022.179(4):727-742.
Beutler LR. GLP-1 physiology and pharmacology along the gut-brain axis. J Clin Invest. 2026.136(2):e194744.

The HbA1c reduction over 30 days should be interpreted with appropriate caveats given that mouse erythrocyte lifespan is approximately 40-45 days. While substantial glycemic improvement over 30 days would be expected to produce measurable HbA1c changes weighted toward the treatment period, the timeframe is less than one complete erythrocyte turnover cycle. The concordance between fasting glucose reduction and HbA1c improvement supports biological plausibility, but this limitation warrants acknowledgment.

Reply:

Thank you for this important point. We acknowledge that the 30-day HbA1c measurements in mice reflect less than a full erythrocyte lifespan (~40–45 days), and therefore the observed reductions should be interpreted with this limitation. We have now noted this limitation in the revised Discussion. Please see **page 15** in the revised manuscript and below:

> "HbA1c changes in mice should be interpreted cautiously, as the 30-day treatment period does not span a full erythrocyte lifespan (~40-45 days)[49]."

The use of oxygen-dependent reporters (GFP, luciferase) for visualizing bacterial activity in the largely anaerobic colonic environment is a technical limitation. While the Extended Data includes microaerobic growth conditions, these do not replicate colonic oxygen tension. The authors should clarify that these imaging data serve as visualization tools and that the primary evidence for in vivo function comes from oxygen-independent endpoints (serum GLP-1, blood glucose, insulin, HbA1c).

Reply:

Thank you for pointing this out. We have accordingly revised the Discussion to clarify that these imaging data are intended for visualization purposes and that the primary evidence for *in vivo* GIFT activity comes from oxygen-independent endpoints, including serum GLP-1, blood glucose, insulin, and HbA1c measurements. Please see **page 15** in the revised manuscript and below:

> "Oxygen-dependent reporters were used only for visualization; therapeutic efficacy was supported by oxygen-independent functional outputs."

The statistical methods section specifies ANOVA with post-hoc tests but does not indicate the specific correction method applied for multiple comparisons. Given the large number of simultaneous statistical tests across the figures (particularly Figure 4 and Figure 6), explicit statement of the multiple comparison correction method is important for reproducibility and interpretation.

Reply:

Thank you for this valuable guidance. When applicable, ANOVA analyses were followed by Tukey's multiple-comparisons test to correct for multiple testing. We have now stated this in the Methods section and in the relevant figure captions. Please see **page 38** in the revised manuscript and

below:

> "When applicable, ANOVA analyses were followed by Tukey's post hoc multiple-comparison test to correct for multiple testing."

The antibiotic resistance markers (kanamycin, ampicillin) used for plasmid selection would require replacement for clinical translation, worthy of acknowledgement at minimum.

Reply:

Thank you for this valuable guidance. We now acknowledge this limitation in the revised manuscript. Please see **page 16** in the revised manuscript and below:

> "The current plasmid-based circuit was useful for facilitating rapid prototyping; however, the prospects for the clinical translation GIFT would be improved by generating cells with stable chromosomal integration of the circuit, as well as removal of antibiotic resistance markers."

Again, we express our sincere gratitude for the helpful guidance in improving our study and manuscript. Many thanks!

Nature 2023-10-18581C: Point-by-point response

Referee #1 (Remarks to the Author):

The authors have addressed all my concerns.

Reply:

We sincerely thank the reviewer for the careful evaluation of our manuscript and for the positive feedback. We are pleased that the reviewer feels all concerns have been adequately addressed.

Referee #3 (Remarks to the Author):

First, I must admit that I am not the best reviewer for this paper. While we have been investigating a microbiome-based approach for diabetes therapy, this project goes far beyond any work I have done in animals. For instance, I am not qualified to address the statistical methods the authors employed. From my initial read, I thought the results Gao and colleague presented to be both fascinating and an encouraging novel new approach in diabetes treatment. The new version of the paper in view addresses the other reviewers' concerns effectively. The paper is ready to be viewed by the world. I think that some of the additional experiment proposed could be addressed in subsequent papers by this team.

Reply:

We sincerely thank the reviewer for the positive evaluation of our work and for the thoughtful comments. We are encouraged that the revised manuscript has satisfactorily addressed the concerns raised during the review process. We are currently conducting additional preclinical studies to further evaluate the safety, efficacy, and translational potential of this therapeutic strategy, with the aim of advancing its future clinical translation.