

A modular synthetic promoter toolkit for programmable gene control in activated and chronically stimulated T cells

Corresponding Author: Dr Velia Siciliano

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Bonfa et al. report the development of a library of novel synthetic promoters (SPs) and comprehensively evaluate their performance in HEK293 and Jurkat cell lines. The analysis is rigorous and convincing. In addition, the authors provide compelling examples of logic gate design and regulatory circuits to achieve high-fold induction of payload delivery in response to an external stimulus. Finally, they validate the utility of one of the best-performing SPs in primary human T cells. While the construction of these novel synthetic promoters provides versatile tools for engineering T cells for various applications, the significance of the study is limited by insufficient evidence supporting its translational application in vivo. Additionally, some technical concerns weaken the strength of the study.

Major Comments:

1. Although the novel SP library developed by the authors could be useful for genetically engineering T cells to advance cancer immunotherapy, the overall motivation of the study is not clearly defined. What major challenge does this study aim to address? The authors should clarify their objectives in the Introduction. If the goal is to develop a new set of SPs that can sense T cell activation (as exhaustion is also a stage of activation), the authors should compare their SPs with existing T cell activation promoters, such as NFAT and NR4A promoters, and highlight the advantages of their approach. If the goal is to develop SPs that are specific to the exhaustion stage—an area where no such SPs have been reported but are highly needed—the authors should focus on validating their SPs under exhaustion-promoting conditions, such as chronic infection or cancer models in vivo. While the study appears to aim at generating synthetic promoters responsive to transcription factors (TFs) that are upregulated in exhausted T cells, the seven selected TFs are reported to be expressed in both activated and exhausted T cells. To exclusively target the exhaustion stage, the authors should consider selecting TFs that are uniquely expressed in exhausted T cells, such as TOX, which has recently been identified as a hallmark of T cell exhaustion. If incorporating TOX-responsive SPs is not feasible, the authors should discuss the reasons behind this choice.
2. Although the SP library is intended for use in T cells, the ON-OFF state characterization and also the following circuit design appear to perform significantly better in HEK293 cells than in Jurkat cells. This raises concerns about transfection efficiency and system robustness in T cells. It is likely that the electroporation efficiency in Jurkat cells was suboptimal. For instance, most IRF4/BATF-sensing SPs that functioned in HEK293 cells failed to work in Jurkat cells. The authors should provide data on the transfection efficiency of each plasmid when co-electroporating three plasmids (pSP, pCMV-TF, and pCMV-EGFP) into Jurkat cells, particularly for pCMV-TF, which is likely larger in size. A low electroporation efficiency of pCMV-TF could significantly impact the absolute induction levels.
3. The transfer learning analysis (Figures S18, S23, and 2K) is interesting but appears somewhat redundant in this study. The prediction results do not align perfectly with the experimental characterization, and subsequent parts of the study do not rely on conclusions drawn from this analysis. While this machine learning-based approach could have a substantial impact in the field, its inclusion in this study does not seem essential.
4. The orthogonality of SPs has not been tested or discussed. Since the consensus sequences of TF binding sites (TF-BSs) may be shared by different TFs, the authors should test whether there are cross-reactivities among SPs when introducing different master TFs in T cells. At a minimum, mutual cross-reactivity among the seven selected TFs in relation to the best-performing SPs should be experimentally ruled out.
5. Based on the histogram in Figure 5E, the NR4A2 level in chronically stimulated T cells appears to be less than fivefold higher than in activated T cells. However, the induction of SP73-mCherry in chronically stimulated T cells seems to be over an order of magnitude higher. To clarify this discrepancy, the authors should examine the correlation between NR4A2 expression and mCherry induction in the same flow cytometry plot and provide a statistical summary of this data.

Minor Comments:

1. In Figures S1 and S2, the differential expression of each TF in activated and exhausted T cells is difficult to discern due to image resolution. It would be helpful to indicate or summarize the expression fold changes of these TFs in activated/exhausted states compared to the naïve state.
2. The absolute induction values shown in Figures 2A-B appear to be calculated differently from other figures. The authors should clarify the calculation method in the figure legend or standardize it across all figures. Additionally, the calculation of mCherry geometric mean fluorescence intensities (gmFIs) in Figure 1 is inconsistent with the description in lines 596–597.
3. The authors should discuss the rationale behind the number of TF-BS repetitions. Why do some SPs have only three TF-BS repeats? In some cases, is five repetitions necessary for achieving higher induction, or does a lower number of repeats help reduce background noise?
4. In line 233, it appears that the reason IKZF2 SPs did not show functional responses is due to low induction in the ON state rather than high noise in the OFF state, as all absolute inductions are below 0.01.
5. In Figures S22E-H, the quantification of CCL21 appears problematic. How is it possible that induced CCL21 production is detected while the constitutive expression in the positive control is undetectable in both cell lines?
6. The schematics in Figures S6A, S7A, S8A, S9A, S13A, S14A, S15A, and S16A should be modified to display only the core promoters used in the corresponding figures rather than showing all four promoters.
7. The methods section lacks details on the construction of different pCMV-TF plasmids, as well as specific information such as Addgene IDs or UniProt IDs for the TFs.
8. In line 617, "penicillin/streptomycin" is mentioned redundantly and should be corrected.

Reviewer #2

(Remarks to the Author)

Summary

This manuscript describes a library for engineering synthetic promoters that are responsive to transcription factors associated with T cell activation and exhaustion. The authors combine minimal promoters with repetitive TFBSs to demonstrate promoter activity in HEK293 and Jurkat cells, and integrate these promoters into Boolean circuits and noise-reducing motifs. Finally, the NR4A2-responsive SP was validated in primary human T cells. However, the design strategy seems closely related to techniques widely used in existing research and lacks direct comparisons with established synthetic promoters. Moreover, the deep learning model's contribution is not fully substantiated, with its performance metrics and integration with experimental data remaining inadequately demonstrated. Collectively, these issues diminish the overall innovation and significance of the work.

Major comments

1. The synthetic promoters constructed using repetitive TFBSs have been extensively reported in previous studies (e.g., *Cell*, 2017, 171(5): 1138-1150. e15; *Nat. Commun.*, 2019, 10(1): 2880). Recent work (*Nat. Commun.*, 2024, 15(1): 10353) has demonstrated the construction of thousands of synthetic promoters by combining three types of minimal promoters with various repetitive TFBS sequences characterized in HEK293 cells, which is the same cell line used in this study. The manuscript should more prominently emphasize its innovative aspects and key highlights. Moreover, a direct comparison with existing synthetic promoter systems (e.g., *ACS Synth. Biol.*, 2022, 11(4): 1440-1453; *Science*, 2022, 378(6625): 1227-1234) is needed to clearly demonstrate the advantages of the new design.
2. The authors demonstrated the activation of the NR4A2-responsive SP in primary human T cells, highlighting its potential for practical applications. However, a direct comparison with existing synthetic promoters (e.g., NFAT and NR4A-based promoters reported in *ACS Synth. Biol.*, 2022, 11(4): 1440–1453) is necessary to clearly showcase the improvements offered by the new design. Without such comparative data, the purported improvements remain unsubstantiated.
3. Several critical details are missing regarding the deep learning model, such as the source of the ChIP-seq dataset, the composition of the validation set, and overall model performance. Additionally, applying the pre-trained model directly to a small dataset (~80 sequences) without fine-tuning is problematic, as indicated by the modest correlation ($r = 0.49$). This performance makes it difficult to extract reliable and useful insights from the model.
4. Promoter activation is evaluated by co-expressing the corresponding transcription factors with the synthetic promoters. Given that promoter activity is dose-dependent and that variations in coding sequences can lead to differences in protein expression levels in mammalian cells, the resulting TF concentrations may vary significantly among constructs. Consequently, absolute induction values across different TF types may not be directly comparable. It is essential to quantify and normalize TF expression levels to ensure a meaningful functional comparison.
5. The term "noise" is frequently mentioned but is not clearly defined or quantified, particularly with respect to the OFF state of the promoters. A detailed explanation of how noise is calculated, along with its biological significance, is necessary. Moreover, the limited number of experimental replicates (often only two) raises concerns about the statistical robustness of these measurements; additional replicates or more rigorous statistical analysis would improve confidence in the reported values.

Minor comments

1. While the manuscript analyzes transcription factors upregulated during various stages of T cell activation and exhaustion (Supplementary Figs. 1 and 2), a more precise quantitative assessment of TF expression levels would further justify the selection criteria used.
2. In Figure 2B, the no-TF control for the IRF4/BATF group (e.g., SP61-No TF) shows unexpectedly high basal expression, contradicting the narrative in the text. This inconsistency should be thoroughly investigated and clarified.
3. The manuscript contains redundant statements; for instance, lines 266–268 repeat the process of applying the pre-trained network to rank the synthetic promoters based on reporter fluorescence.

4. There are duplicate references—for example, the Wu et al. Nat. Commun. 10, 717 (2019) citation appears twice.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

Manuscript of Bonfa and Siciliano titled "Design of novel synthetic promoters to tune gene expression in T cells."

The study aims to develop the control of transgene expression in cells. Specifically, Chimeric-Antigen-Receptor T cells (CART) are of great clinical interest nowadays, utilizing constitutive strong promoters for transgene expression. Authors envisioned a most interesting change of the method by screening a library of some 80 synthetic promoters that are suggested to respond to seven major transcription factors of activated or exhausted human T cells, namely: IRF4, BATF, MAF, GATA3, NR4A2, EOMES, IKZF2 (also known as IKAROS). The list of TFs was selected from extensive datasets, and filtration according to RNA-seq data, which is already interesting. Notably, some are well known, and some are less considered of CART so far. Known TF-binding sites were cloned as 5-tandem repeats with a minimal promoter (selected from YB-TATA, miniCMV, miniTK, or LateADEp).

Cloned promoters were tested in HEK293 and Jurkat cell lines. That a reasonable start- but must recognize that HEK are not T-cells, and Jurkats cell lines (and subclones) were reported to have impaired signaling. This is perhaps the major limitation of this study. One other limitation to consider is the use of plasmids. CART are currently made with Retro- or Lenti-vectors that integrate into the genome. Using plasmids is legitimate, yet it might differ from viral vectors (it gains a transient expression, not permanent integration as CART is currently being manufactured).

Data showing the expression from synthetic promoters w/o stimulation (authors call it "Background noise" or "leakiness"). Induction of selected promoters with the TFs is brilliantly demonstrated in Figure 2, with the notion of limitation stated above. Authors smartly moved on to design and test more sophisticated synthetic promoters that encompass multiple TFs response elements that are designed for boolean logic gates- as shown in figure 3, each synthetic-promoter drives split-GAL4VP16 transcription factor. This ambitious part is truly remarkable, yet we must also recognize possible limitations- as the biological activity of complicated promoters do not always follow strict logical roles (unfortunately). Authors go further, using the rTA-system to allow additional external control (as published by several studies), demonstrating how their novel promoter may work together with the rTA-DOX system to control the expression w/o the TFs. Finally, primary human CD8 are shown to work with a selected promoter and elevate reporter-gene expression at chronic-stimulation state in vitro; HEK and Jurkat cell lines were shown to secrete IL12 as an example for an interesting effector rather than reporter-gene only.

Overall, the study is very interesting and will be published.

Major comments:

1. Authors must clearly declare limitations, including the above-mentioned work with cell lines and non-integrating plasmids. In the last part, they did use a lentivector for the primary human T cells, perhaps mentioning the correlation between plasmids and LVs if many of these were co-studied.
2. Supplementray data is exceptionally good- consider moving representative raw data (aka FACS plots) of unstimulated and stimulated cells to maintext- so readers can appreciate the quality of your data!
3. This study is focused on the design of novel synthetic promoters for CART. Authors better refer to previous studies that aimed at the same target- and let the reader appreciate the novelty of their approach (which is truly of interest for the broad readership of Nature Communication).
4. Titles of figures captions are somewhat descriptive of intention, data is very good- and authors are encouraged to let some more active caption-titles that highlight their achievements.
5. The last part, demonstrating the activity of the synthetic promoter in primary human T cells following chronic stimulation in vitro, is referred to as "Functionality in primary human T cells upon antigen stimulation" (lines 100-101). However, as far as I can see, these T cells were stimulated using the strong anti-CD3+anti-CD28 beads, not by specific antigens. Please excuse me if I missed something (perhaps data not shown, or my limited understanding); alternatively do change the text to fit with the presented data, which is a strong activation NOT of a specific antigen.

Minor comments:

1. Why did you choose 5Xrepeats?
2. How do you envision utilizing such a transgenic-TFs system in vivo? If rather than let it go with lentivectors and dependence on endogenous TFs, please consider and write expected differences between T-cell subtypes.
3. If you have data of multiple promoters tested as plasmids and as lentivectors, this might be of interest to the broader readership, as your method is not limited to CART.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Bonfá et al. have addressed several of the previously raised issues, including the addition of in vivo validation for the NR4A2 SP in a mouse xenograft model, and the revised Introduction better articulates the intended scope of the study. However, my major concerns remain unresolved, and in my view the revised manuscript still does not provide the level of conceptual advance, rigor, or physiological relevance expected for publication in Nature Communications.

Major point 1

Despite the revised title and Introduction, the manuscript still overstates the breadth and versatility of the proposed toolkit. The study is presented as a set of synthetic promoters responsive to multiple TFs associated with activation and chronic stimulation, offering a spectrum of transcriptional behaviors for distinct applications. However, this claim is not supported by the actual design of the study.

All seven TFs used for SP design are derived from a single T-cell state, namely exhausted or chronically stimulated T cells. This immediately limits the generality of the proposed toolkit. TF activity differs substantially across naïve, effector, memory, and exhausted T-cell states, and these states are governed by distinct regulatory networks. A genuinely versatile promoter toolkit would therefore require responsive elements linked to TFs active across multiple, biologically distinct T-cell states. In its current form, the manuscript does not establish such breadth.

More importantly, only the NR4A2 SPs were evaluated under endogenous TF regulation. The SPs responsive to the other six TFs were tested only in the context of exogenous TF overexpression. Their behavior within native T-cell regulatory circuits therefore remains entirely uncharacterized. This is not a minor gap; it directly undermines the manuscript's core claim that these promoters can harness endogenous TF dynamics for programmable control in T cells.

Major point 2

The authors repeatedly state in the response letter that their goal is neither to benchmark against prior NR4A-based systems nor to develop exhaustion-specific SPs, but rather to establish a modular SP toolkit. However, the work ultimately does not deliver a substantively new use case beyond antigen-responsive NR4A SP activation. That application has already been reported and is conceptually very similar to prior NR4A-based systems, as well as to more established NFAT-based activation-responsive promoters.

This limited advance is a serious problem for a journal at this level. Although the manuscript includes screening of numerous SPs, the downstream validation converges almost entirely on a single promoter class that is already comparatively well developed in the literature. As a result, the study does not convincingly establish either a broadly enabling platform or a clear conceptual step beyond prior work.

Major point 3

I appreciate that the authors quantified TF mRNA levels in Jurkat cells to assess electroporation performance. However, I do not agree with the conclusion that "while activation amplitudes differed between cell types, consistent with reduced TF availability, the relative behavior of many promoters was conserved." Based on the presented data, at least half of the SPs, particularly those associated with BATF, EOMES, and IKZF2, show altered relative behaviors compared with other members of the same TF cluster.

It therefore remains unclear whether these differences reflect poor transfection efficiency, broader technical variability, or genuine cell-type-specific regulatory effects. This uncertainty is important, because it directly affects interpretation of promoter behavior in the more relevant T-cell context. A more rigorous strategy would have been to incorporate a fluorescent reporter such as BFP into the TF-expression plasmids, analogous to the dual-promoter constructs used in primary T cells, and then gate on BFP-positive cells, or preferably BFP+GFP+ double-positive cells if the SP vectors carry a constitutive GFP cassette. That approach would have allowed a much more reliable comparison of mCherry MFI and would have substantially strengthened the Jurkat data. In the absence of such control, the conclusions drawn from these experiments remain insufficiently supported.

Minor points

1. Figure S2 and TF selection.

The genes in Fig. S2 were clustered using Euclidean distance, but no dendrogram or equivalent visualization is provided. It is therefore difficult to assess whether the seven chosen TFs truly emerge from distinct clusters or whether they capture meaningfully different transcriptional behaviors during T-cell differentiation. This information would be important for understanding the logic of TF selection and for guiding future use of the proposed SPs.

2. Orthogonality analysis.

The orthogonality matrix is generally informative and supports the conclusion that cross-activation by non-cognate TFs is limited, with NR4A2 SPs appearing to perform best overall. However, GATA3 SPs are absent from Fig. 2K. In addition, because the manuscript is explicitly focused on T-cell applications, an orthogonality matrix generated in Jurkat cells would be more relevant and more convincing than one derived primarily from HEK293 cells.

3. TOX-related SPs.

The fact that TOX is only weakly upregulated under persistent stimulation does not, by itself, argue against the possible utility of a TOX-responsive SP. One of the advantages of synthetic promoter design is that repeated core motifs can amplify transcriptional responses even when endogenous TF abundance is modest. It would therefore be helpful to indicate where TOX ranks within the transcriptomic datasets used for TF selection in Figures S1 and S2.

4. CCL21 quantification.

The issue with CCL21 quantification remains unresolved. If the authors cannot explain, or reproducibly confirm, why constitutive expression yields lower CCL21 output than SP73, then these data should be removed, as they currently risk misleading interpretation. Alternatively, the experiment should be repeated with a different therapeutic cargo for which expression can be quantified more robustly.

5. SP91 preparation.

The preparation of SP91 is not described in the Methods or supplementary tables. This omission should be corrected.

6. Chronic stimulation protocol.

The description of the chronic stimulation protocol using Dynabeads remains unclear. Specifically, the phrase "repeated stimulation every 48 h" needs to be defined explicitly. Were the Dynabeads continuously present, or were they removed and reintroduced at each cycle? This distinction is important for interpreting the stimulation regime.

7. Presentation and typographical errors.

The manuscript still contains multiple typographical and presentation issues that should have been resolved at the revision stage.

In the merged main text and figure file:

Line 511: stimulation

Line 539: at

Line 551: mCherry

Line 554: engineered

Line 583: Leukocyte

Lines 656 and 671: promoters

Fig. 7H: TEX

In the supplementary file:

Line 152: Absolute

Fig. S20: pCMV

Overall, while the revision improves some aspects of the manuscript, the central concerns remain: the platform claim is overstated, the experimental validation is too narrow, and the most physiologically relevant systems are not examined with sufficient rigor. For these reasons, I remain unconvinced that the manuscript reaches the level of novelty and completeness required for Nature Communications.

Reviewer #2

(Remarks to the Author)

The authors have addressed all of our comments.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

Thanks for the extensive revisions and the additional data. The revised version substantially enhances the paper.

I do find the paper adequate to publish.

Realizing other reviewers had some less favorable comments and overall appreciation, I must note the study is original, interesting, and will have an impact. It is, in my opinion, not fair to dismiss such a good study because other related papers are published- this is true for >90% of good papers, and we must publish solid research that brings new data, even if related studies are present. Basic, fundamental science must have a place to publish and communicate findings that will progress our society.

I encourage the authors to get it published as it should be.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have further revised the manuscript and performed additional experiments to address the concerns raised during the previous round of review. They have now satisfactorily addressed major comments 1 and 3, as well as all of my

previous minor comments.

However, I remain only partially convinced by the authors' response to major comment 2 concerning novelty. Although the manuscript now provides a more comprehensive in vivo characterization of NR4A2-based synthetic promoters (SPs) than previous studies, these advances are still focused on an already established SP. Other SPs beyond the NR4A- and NFAT-based systems, which are likely to be of considerable interest to the community, were neither characterized to a comparable extent nor directly benchmarked against the NR4A2-based SP. While I appreciate that the promoter architectures presented here could be readily adapted for applications beyond T-cell engineering, the manuscript would provide substantially greater value to the field if the authors deposited a plasmid toolkit containing the principal SP constructs described in the study in a publicly accessible repository.

Minor comments

1. Figure 7H: Exhausted T cells are conventionally abbreviated as TEX rather than TTEX. Alternatively, if the authors prefer the term "dysfunctional T cells," an abbreviation such as Tdysf would be appropriate. Regardless of the terminology chosen, the full term and its abbreviation should be introduced clearly and used consistently throughout the manuscript.
2. The description of Figure 7H in the main text is not fully supported by the data presented. Specifically, Figure 7H does not appear to show an analysis of the relationship between mCherry expression and exhaustion-marker expression. Furthermore, the gating strategy used to identify exhausted T cells within the tumour should be described in sufficient detail. The statement that "multiple exhaustion markers were combined to determine..." is too vague and should be replaced with a precise description of the markers used and the gating strategy applied.
3. Details of the antibodies used to detect the endogenous transcription factors MAF, IRF4, BATF, GATA3, IKZF2, and EOMES in primary T cells are missing and should be included in the Methods or Supplementary Information.

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

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

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

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

We are grateful to the reviewers for their careful evaluation and constructive comments, which have helped us improve the quality and completeness of the manuscript. We have carefully addressed each point raised and performed substantial additional analyses and experiments where appropriate. Below, we provide a detailed point-by-point response, with corresponding revisions incorporated into the manuscript.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Bonfa et al. report the development of a library of novel synthetic promoters (SPs) and comprehensively evaluate their performance in HEK293 and Jurkat cell lines. The analysis is rigorous and convincing. In addition, the authors provide compelling examples of logic gate design and regulatory circuits to achieve high-fold induction of payload delivery in response to an external stimulus. Finally, they validate the utility of one of the best-performing SPs in primary human T cells. While the construction of these novel synthetic promoters provides versatile tools for engineering T cells for various applications, the significance of the study is limited by insufficient evidence supporting its translational application *in vivo*.

We thank the reviewer for the positive assessment of our work. We agree that the primary contribution of this study is the development of a versatile and rigorously characterized synthetic promoter (SP) platform, which expands the available toolkit for engineering cells with transcriptional control. The scope of the present manuscript is intentionally focused on establishing and validating this platform, including comprehensive *in vitro* characterization, circuit implementation, and proof-of-concept testing to check the suitability prior primary T cell engineering. This would enable *in vitro* application such as real-time monitoring of T cell functional states and facilitate the screening of pharmacological or genetic interventions that modulate T activity to gain deeper understanding of the mechanisms underlying cell response. We believe this foundation is essential before advancing toward *in vivo* applications. Nevertheless, we fully recognize the importance of translational relevance.

Although *in vivo* validation was not the primary scope of this study, we have taken initial steps in this direction by evaluating the best-performing NR4A2-responsive promoter (SP73) in an adoptive T cell transfer setting. In collaboration with Professor Luca Gattinoni's group (Leibniz Institute for Immunotherapy, University of Regensburg), we assessed SP73 activity in NY-ESO-1–TCR CD8⁺ T cells in a humanized melanoma xenograft model. Tumor-infiltrating T cells exhibited detectable SP73-driven reporter expression, indicating that the promoter can be activated *in vivo* within the tumor microenvironment. Importantly, reporter activity decreased following tumor regression, consistent with reduced NR4A2 levels and supporting the reversible and stimulation-dependent behavior observed *in vitro*.

Collectively, these observations support the translational potential of the SP platform presented here and suggest that SP73 can function as an *in vivo* sensor of NR4A2-driven transcriptional activity. We view these results as encouraging preliminary evidence and a

strong motivation for future studies aimed at systematic in vivo evaluation and therapeutic implementation. The data are included in the revised manuscript as new **Figure 7**.

Additionally, some technical concerns weaken the strength of the study.

Major Comments:

1. Although the novel SP library developed by the authors could be useful for genetically engineering T cells to advance cancer immunotherapy, the overall motivation of the study is not clearly defined. What major challenge does this study aim to address? The authors should clarify their objectives in the Introduction. If the goal is to develop a new set of SPs that can sense T cell activation (as exhaustion is also a stage of activation), the authors should compare their SPs with existing T cell activation promoters, such as NFAT and NR4A promoters, and highlight the advantages of their approach.

We thank the reviewer for this comment, which allows us to clarify the motivation and scope of our study.

The aim of this work is to develop and characterize a library of synthetic transcriptional sensors that respond to transcription factors associated with T cell activation and persistent antigen stimulation. By linking promoter activity to endogenous TF expression, these synthetic promoters provide a modular tool to modulate or report gene expression changes that occur as T cells experience different antigen-stimulation conditions. While persistent stimulation is often associated with T cell dysfunction or exhaustion, our study does not aim to define, classify, or selectively target specific functional states. Instead, we focus on engineering and systematically evaluating TF-responsive synthetic promoters in terms of basal activity, inducibility, and suitability for integration into genetic circuits.

We have revised the Introduction accordingly to better reflect this objective and to clearly position the study as a platform-oriented and exploratory contribution.

With respect to existing activation-responsive promoters such as NFAT- or NR4A-based systems, these represent well-established approaches for sensing acute TCR signaling. Our work is not intended to benchmark or outperform these promoters; rather, we expand the available design space by providing a toolkit of synthetic promoters responsive to multiple TFs linked to activation and chronic stimulation, offering a range of transcriptional behaviors that can be selected according to experimental or application-specific needs.

However, as suggested by the reviewer, we have carried out a comparison with an NR4A promoter previously described by Guo et al. (2022), since NR4A2 is included among the transcription factors targeted in our library. A direct comparison between NFAT- and NR4A-based promoters has already been reported in that study, and therefore we did not repeat this analysis here.

Direct comparison with the NR4A promoter described by Guo et al. (2022) demonstrates that SP73 achieves higher inducible activity under identical experimental conditions in both HEK293 and Jurkat cells (New **Supplementary Figure S24A-B**). However, we would like to clarify that this comparison is intended to place SP73 in the context of existing NR4A-responsive designs rather than to establish a general benchmark, since the goal of our study is not to identify a single promoter that universally outperforms previously reported systems, but to provide a set of synthetic promoters spanning an operational range of transcriptional behaviors. This enables

the selection of an SP with properties that best match the requirements of a given downstream application.

If the goal is to develop SPs that are specific to the exhaustion stage—an area where no such SPs have been reported but are highly needed—the authors should focus on validating their SPs under exhaustion-promoting conditions, such as chronic infection or cancer models *in vivo*.

We thank the reviewer for this important comment. We would like to clarify that the aim of the current work is not to develop synthetic promoters that are specific to the exhaustion stage *per se*, but rather to provide a set of transcriptional sensors that respond to transcription factors associated with T cell activation and persistent stimulation, thereby offering a range of operational behaviors in T cells.

We agree that evaluating promoter activity under exhaustion-promoting conditions is informative. For this reason, we performed additional experiments in primary human CD8⁺ T cells using the most effective NR4A2-responsive synthetic promoters identified in our library (SP73, SP74, SP75, SP76, SP77, SP78). Due to practical limitations associated with primary T cell availability, we did not test the full library and instead focused on the most robust candidates for further *in vitro* characterization.

We first established an **in vitro model of persistent stimulation** by repeatedly activating CD8⁺ T cells with anti-CD3/CD28 dynabeads. While this model does not fully recapitulate chronic infection or tumor-driven exhaustion *in vivo*, it can capture key features associated with T cell dysfunction, including sustained stimulation and upregulation of markers such as PD-1, CD39, TIGIT, and LAG-3.

We recapitulate below our findings, which are now included in the revised manuscript as new **Figures 5, Supplementary Figures S26-S31**.

1. CD8⁺ T cells transduced with NR4A2-responsive SPs driving mCherry expression were cultured for 8 days under either acute stimulation (single stimulation followed by rest) or repeated stimulation using CD3/CD28 dynabeads. Upon withdrawal of stimulation in the acute condition, mCherry expression declined over time, whereas repeated stimulation induced a progressive and sustained increase in reporter activity, paralleling endogenous NR4A2 expression dynamics (new **Figure 5B-C**). Among the tested variants, SP73 and SP78 showed the highest induction, in line with what observed in HEK293 and Jurkat cells. SP75 exhibited similar fraction of mCherry⁺ cells compared to SP75 and SP78 (**Supplementary Figure S26A-B**), but a lower degree of mCherry expression in terms of fluorescent intensity (**Supplementary Figure S26C**), illustrating how promoter architecture influences responsiveness. In parallel, markers associated with persistent stimulation were increased under chronic conditions (new **Figure 5D**).
2. Because the dynamic ON-OFF activation is an important feature of inducible promoters, we investigated whether NR4A2-responsive SP73 and SP78 could switch OFF following removal of the stimulus. Upon dynabeads withdrawal, mCherry expression gradually decreased, indicating promoter deactivation (**Figure 5F, Supplementary Figure S28A**). Re-exposure to stimulation resulted in renewed reporter induction, demonstrating that

SP73 and SP78 activity is dynamic and reversible under these conditions (**Figure 5G**, **Supplementary Figure S28B**).

3. Finally, we evaluated SP73 activity in CD8⁺ T cells expressing an NY-ESO-1-specific TCR and repeatedly exposed to NY-ESO-1⁺ melanoma cells in vitro. Using live-cell imaging and flow cytometry, we observed progressive increases in mCherry expression over tumor cell exposure, consistent with increased NR4A2 expression (**Figure 6B-D**). In parallel, markers associated with persistent stimulation increased, while cytokine production and cytotoxic markers decreased over time (**Figure 6D**). In contrast, T cells engineered with a control synthetic promoter lacking NR4A2 binding sites (SP01) did not exhibit reporter activation (**Figure 6B-C**).

Collectively, these in vitro data indicate that NR4A2-responsive synthetic promoters, particularly SP73, function as dynamic and reversible transcriptional sensors that reflect changes in transcriptional programs associated with sustained T cell stimulation.

While the study appears to aim at generating synthetic promoters responsive to transcription factors (TFs) that are upregulated in exhausted T cells, the seven selected TFs are reported to be expressed in both activated and exhausted T cells. To exclusively target the exhaustion stage, the authors should consider selecting TFs that are uniquely expressed in exhausted T cells, such as TOX, which has recently been identified as a hallmark of T cell exhaustion. If incorporating TOX-responsive SPs is not feasible, the authors should discuss the reasons behind this choice.

We agree with the reviewer that a synthetic promoter responsive to a transcription factor uniquely upregulated in exhausted T cells would, in principle, enable a more restricted and specific response. However, identifying such a TF that is both exhaustion-specific and amenable to SP targeting remains challenging. Further, our goal in this study was not to generate exhaustion-specific promoters, but rather to develop a modular library of transcriptional sensors responsive to transcription factors associated with T cell activation and persistent stimulation.

With respect to TOX, we acknowledge that it has been described as a key regulator and marker of T cell exhaustion. Following the reviewer's suggestion, we examined TOX expression in our in vitro model of acute and persistent CD3/CD28 stimulation using primary human CD8⁺ T cells. We observed that TOX expression increases upon activation, with only a modest additional increase under chronic stimulation conditions (**New Supplementary Figure S25**), consistent with previously published reports¹. Importantly, absolute TOX expression levels in our dataset were relatively low compared to other transcription factors included in this study, such as NR4A2 (**Supplementary Table S1**).

From a promoter-engineering perspective, effective synthetic promoter activation depends not only on differential expression but also on sufficient transcription factor abundance and well-characterized binding motifs suitable for multimerization. Given the modest differential increase and relatively low expression levels observed for TOX in our system, we did not prioritize it for SP construction in the current study.

Instead, to achieve greater control and context selectivity, we explored combinatorial transcriptional strategies, including logic-gate architectures and network-based circuit

topologies (Figures 3 and 4), which allow transcriptional output to be restricted by integrating multiple TF inputs rather than relying on a single exhaustion-associated factor. We have clarified this rationale in the revised manuscript.

2. Although the SP library is intended for use in T cells, the ON-OFF state characterization and also the following circuit design appear to perform significantly better in HEK293 cells than in Jurkat cells. This raises concerns about transfection efficiency and system robustness in T cells. It is likely that the electroporation efficiency in Jurkat cells was suboptimal. For instance, most IRF4/BATF-sensing SPs that functioned in HEK293 cells failed to work in Jurkat cells. The authors should provide data on the transfection efficiency of each plasmid when co-electroporating three plasmids (pSP, pCMV-TF, and pCMV-EGFP) into Jurkat cells, particularly for pCMV-TF, which is likely larger in size. A low electroporation efficiency of pCMV-TF could significantly impact the absolute induction levels.

We thank the reviewer for this important and insightful comment. It prompted us to more carefully examine the differences observed between HEK293 and Jurkat cells during transient screening. In our transient delivery experiments, we routinely include a constitutively expressed fluorescent marker (EGFP) to monitor transfection or electroporation efficiency. As expected, we observed greater variability in electroporation efficiency and viability in Jurkat cells compared to HEK293 transfections. Nonetheless, electroporation efficiency and cell viability were consistently within a range that allowed reliable downstream analysis.

However, as the reviewer correctly notes, expression of a fluorescent marker does not necessarily reflect equivalent expression of co-delivered plasmids, particularly when multiple plasmids are co-electroporated. To address this concern directly, we performed additional experiments to quantify TF expression levels following transfection (HEK293) and electroporation (Jurkat). Using qPCR analysis, we observed substantially lower TF expression levels in Jurkat cells compared to HEK293 cells (**Supplementary Figure S35**). For example, in the case of IRF4/BATF, TF expression levels were approximately two orders of magnitude lower in Jurkat cells. While mRNA quantification does not directly measure protein abundance, the consistent ranking of promoter activation across cell types and delivery systems supports the conclusion that reduced TF availability, rather than loss of promoter specificity, accounts for the lower induction observed in Jurkat cells. These findings support the reviewer's hypothesis that reduced TF expression contributes to the lower absolute induction observed in Jurkat cells. Importantly, while activation amplitudes differed between cell types, consistent with reduced TF availability, the relative behavior of many promoters was conserved.

To further assess whether the transient screening reflects biologically relevant behavior in T cells, we extended the analysis to primary human CD8⁺ T cells using lentiviral integration. We tested six NR4A2-responsive promoters (SP73–SP78). In primary T cells stimulated with anti-CD3/CD28 dynabeads, SP73 and SP78 showed robust activation and dynamic ON–OFF–ON behavior (**Figure 5; Supplementary Figures S26–S28**). SP75 was activated at lower levels, while SP74, SP76, and SP77 did not show detectable reporter induction (**Supplementary Figures S26**). Notably, this relative ranking mirrors the behavior observed in transient screening experiments. Together, these results indicate that although transient plasmid-based screening in Jurkat cells has inherent limitations—particularly with respect to reduced TF expression levels—the combined screening approach in HEK293 and Jurkat cells remains informative for prioritizing

promoter architectures that retain functionality following lentiviral integration in primary T cells. We have clarified these points in the revised manuscript and now explicitly discuss the methodological limitations, interpreting the Jurkat data with appropriate caution.

3. The transfer learning analysis (Figures S18, S23, and 2K) is interesting but appears somewhat redundant in this study. The prediction results do not align perfectly with the experimental characterization, and subsequent parts of the study do not rely on conclusions drawn from this analysis. While this machine learning-based approach could have a substantial impact in the field, its inclusion in this study does not seem essential.

We thank the reviewer for this thoughtful comment. We agree that the modest correlation between predicted and experimentally measured promoter rankings does not support strong claims of model generalizability between the external ChIP-seq dataset and the T-cell promoter library used in this study.

The purpose of including the transfer learning analysis was not to derive biological conclusions that drive subsequent experiments, but rather to explore whether promoter ranking could be partially inferred from externally trained models in situations where the dataset of interest is too small to support de novo model training. In many synthetic biology applications, promoter libraries are limited in size, making traditional supervised machine learning approaches impractical. We therefore evaluated whether a pre-trained model could capture relative trends in promoter strength without fine-tuning.

The ChIP-seq dataset used for model training and internal validation was obtained from the Eukaryotic Promoter Database, where curated datasets have been assembled from published studies. The ~80-sequence T-cell promoter dataset was treated strictly as an independent test set, and no fine-tuning was performed using these data. We have clarified this training-validation-test schema in the revised manuscript to ensure transparency.

We acknowledge that the predictive performance is moderate and does not substitute for experimental characterization. Accordingly, we present this analysis as exploratory and methodological rather than as a central claim of predictive capability. The core conclusions of the study remain fully supported by experimental data independent of the transfer learning analysis.

4. The orthogonality of SPs has not been tested or discussed. Since the consensus sequences of TF binding sites (TF-BSs) may be shared by different TFs, the authors should test whether there are cross-reactivities among SPs when introducing different master TFs in T cells. At a minimum, mutual cross-reactivity among the seven selected TFs in relation to the best-performing SPs should be experimentally ruled out.

We thank the reviewer for raising this important point regarding promoter orthogonality. To address potential cross-reactivity among transcription factors, we performed an orthogonality analysis in HEK293 cells using the best-performing synthetic promoters (SPs) representing each TF (YB_TATA configuration) and systematically co-expressed each SP with all seven transcription factors evaluated in this study. Promoter activity was quantified under identical experimental conditions.

The resulting orthogonality matrix (now included as **Figure 2K and Supplementary Figure S36**) shows that activation is predominantly observed with the cognate transcription factor for each SP. Cross-activation by non-cognate TFs was generally minimal. In a limited number of cases, low-level activation (<5-fold induction relative to baseline) was observed upon MAF overexpression. However, this activation remained substantially lower than the induction achieved with the cognate TF (except for IKFZ2 which showed poor activation) and did not alter the relative ranking or functional interpretation of the promoters.

Overall, these data indicate that the synthetic promoters display a high degree of functional specificity within the tested TF panel. We have added the orthogonality matrix and clarified this analysis in the revised manuscript.

5. Based on the histogram in Figure 5E, the NR4A2 level in chronically stimulated T cells appears to be less than fivefold higher than in activated T cells. However, the induction of SP73-mCherry in chronically stimulated T cells seems to be over an order of magnitude higher. To clarify this discrepancy, the authors should examine the correlation between NR4A2 expression and mCherry induction in the same flow cytometry plot and provide a statistical summary of this data.

We thank the reviewer for this careful observation. We agree that, based on the histogram shown in Figure 5E (of the previous version of this manuscript), the increase in NR4A2 protein levels between activated and chronically stimulated T cells appears modest, whereas SP73-driven mCherry expression shows a larger dynamic range. This apparent discrepancy likely reflects differences between TF abundance and transcriptional output, as well as the intrinsic properties of the synthetic promoter architecture. In particular, SP73 contains multiple NR4A-responsive binding sites, which may amplify transcriptional output in a non-linear manner once a threshold level of NR4A2 activity is reached. In addition, differences in protein stability and accumulation between NR4A2 and mCherry may further contribute to the observed divergence in fold change. Further, transcriptional activation is not expected to scale linearly with transcription factor abundance. Synthetic promoters containing multiple copies of cognate binding sites, such as SP73, are designed to operate under cooperative and threshold-dependent activation regimes, in which small changes in TF levels can result in disproportionate changes in transcriptional output once a critical occupancy threshold is reached. Such non-linear behavior is a well-recognized feature of both endogenous and synthetic promoters and reflects cooperative TF binding, recruitment of transcriptional machinery, and integration of TF activity over time. As a result, differences in fold change between TF protein levels and reporter expression do not necessarily indicate a discrepancy but rather are consistent with the expected properties of multi-site promoter architectures.

As suggested by the reviewer, we examined the relationship between NR4A2 expression and SP73-driven mCherry expression by analyzing both signals within the same flow cytometry dataset. This analysis shows a positive association between NR4A2 levels and mCherry expression, supporting the interpretation that SP73 activity reflects NR4A2-driven transcriptional dynamics rather than absolute TF abundance (new **Figure 5B-D**).

Importantly, we do not claim a linear correspondence between NR4A2 protein levels and reporter output. Instead, these data indicate that SP73 functions as a sensitive transcriptional

sensor whose output integrates NR4A2 activity over time, resulting in an expanded dynamic range relative to the measured TF expression levels.

Minor Comments:

1. In Figures S1 and S2, the differential expression of each TF in activated and exhausted T cells is difficult to discern due to image resolution. It would be helpful to indicate or summarize the expression fold changes of these TFs in activated/exhausted states compared to the naïve state.

We thank the Reviewer for this observation. As described in the *Materials and Methods* section (“Transcription Factors (TFs) and TF-Binding Sites (TF-BSs) selection”), our TF selection was based on the identification of 197 transcription factors commonly upregulated in dysfunctional and effector compared to naïve T cells across multiple publicly available datasets. Because the datasets considered derive from independent large-scale studies with heterogeneous experimental settings and varying definitions of activation and exhaustion sub-states, we chose to represent absolute expression levels rather than fold changes in Figures S1 and S2. Our rationale was to prioritize TFs that were not only upregulated, but also expressed at sufficiently high levels, as reflected by their position in the mid-to-high range of the heatmaps. For this reason, Figures S1 and S2 display TF expression values as $\log_2$ TPM (transcripts per million).

Following the Reviewer’s suggestion, we have now clarified in the figure captions that the heatmaps represent expression values rather than fold changes. In addition, we have updated the Supplementary Table 1, including new spreadsheets reporting the underlying TF expression values used to generate these figures, to facilitate interpretation.

2. The absolute induction values shown in Figures 2A–B appear to be calculated differently from other figures. The authors should clarify the calculation method in the figure legend or standardize it across all figures. Additionally, the calculation of mCherry geometric mean fluorescence intensities (gMFIs) in Figure 1 is inconsistent with the description in lines 596–597.

We thank the reviewer for pointing this out. In Figures 2A–B, which summarize the best-performing synthetic promoters for each transcription factor, we used a simplified calculation based on the mCherry geometric mean fluorescence intensity and the percentage of mCherry-positive cells within the transfected cell population. This approach was adopted to provide a clear and intuitive representation of promoter activity for comparison purposes in this summary figure. We have now explicitly clarified this calculation method in the corresponding figure legend and in the *Materials and Methods* section of the revised manuscript. In addition, we have corrected the inconsistencies related to the calculation and description of mCherry geometric mean fluorescence intensity (gMFI) in Figure 1 and aligned the figure legend and text (lines 596–597).

3. The authors should discuss the rationale behind the number of TF-BS repetitions.

Why do some SPs have only three TF-BS repeats? In some cases, is five repetitions necessary for achieving higher induction, or does a lower number of repeats help reduce background noise?

We thank the reviewer for the opportunity to clarify the rationale behind the number of TF-binding site (TF-BS) repetitions.

Based on prior synthetic promoter studies and our laboratory experience, we initially selected five tandem TF-BS repeats as a design compromise expected to support cooperative TF binding and robust inducible activity while maintaining manageable promoter length. Multimerization of TF-BSs is commonly required to achieve measurable activation from minimal promoters, and five repeats^{2,3} are frequently used in synthetic promoter design to balance inducibility and background activity.

During cloning, however, we observed that certain TF-BS sequences were prone to recombination or instability when assembled in 5× tandem arrays, resulting in constructs containing fewer repeats (typically 3×). Rather than discarding these variants, we retained and tested them to evaluate whether repeat number systematically affected promoter behavior.

Where direct comparisons were possible (e.g., SP65A/B and SP72A/B), we did not observe a consistent correlation between TF-BS copy number and OFF-state leakiness. For example, SP65A (<5× TF-BS) exhibited lower basal activity than SP65B (5× TF-BS), whereas SP72A (<5×) and SP72B (5×) displayed comparable leakiness (**Supplementary Figure S3B**). Similarly, absolute induction levels in the presence of the cognate TF were comparable between 3× and 5× configurations (**Supplementary Figure S6B** for SP65A/B and **Supplementary Figure S9B** for SP72A/B).

These observations suggest that, within the limited comparisons available, TF-BS copy number alone does not predict basal activity or inducibility in a straightforward manner. We have clarified this rationale and included these comparisons in the revised manuscript.

4. In line 233, it appears that the reason IKZF2 SPs did not show functional responses is due to low induction in the ON state rather than high noise in the OFF state, as all absolute inductions are below 0.01.

We thank the reviewer for pointing this out. The reviewer is correct that, for IKZF2-responsive synthetic promoters, the lack of functional response is primarily due to low inducible activity in the ON state rather than high background noise in the OFF state, as reflected by the low absolute induction values. We have corrected the corresponding statement in the manuscript to accurately reflect this observation. We note that the values discussed are expressed in relative units normalized to the EGFP transfection control, which further emphasizes the limited inducibility of the IKZF2 SPs under the tested conditions. By contrast, elevated background activity in the OFF state was observed for GATA3-responsive SPs in Jurkat cells, as described elsewhere in the manuscript.

These clarifications have been incorporated into the revised version of the manuscript.

5. In Figures S22E-H, the quantification of CCL21 appears problematic. How is it possible that induced CCL21 production is detected while the constitutive expression in the positive control is undetectable in both cell lines?

We thank the reviewer for raising this important point. We agree that the apparent absence of detectable CCL21 secretion from the constitutive control warrants clarification.

The plasmid used for constitutive CCL21 expression was sequence-verified, and we confirmed that the same constitutive promoter (UbC) successfully drove detectable IL12p70 expression under identical experimental conditions. This indicates that the construct backbone and delivery method were functional.

However, CCL21 secretion levels were overall modest in our system, including under SP-driven inducible conditions. ELISA measurements for CCL21 were close to the lower detection range of the assay, which likely reduced the sensitivity to detect small differences between constitutive and inducible conditions. Importantly, SP-driven CCL21 expression was consistently detectable above background in the presence of NR4A2 in HEK293 cells, whereas the constitutive control remained near baseline under the same experimental conditions.

We have clarified these points in the revised manuscript and explicitly indicate the detection range and interpretation limits of the ELISA assay to avoid potential misinterpretation of the CCL21 quantification.

6. The schematics in Figures S6A, S7A, S8A, S9A, S13A, S14A, S15A, and S16A should be modified to display only the core promoters used in the corresponding figures rather than showing all four promoters.

We have changed the figures in the new version of the manuscript as per reviewer suggestion.

7. The methods section lacks details on the construction of different pCMV-TF plasmids, as well as specific information such as Addgene IDs or UniProt IDs for the TFs.

We thank the reviewer for pointing out this missing information. Most of the TF expression constructs used in this study were cloned into the pCDNA3.1(+) backbone. Some constructs were generated in-house, while others were obtained from Addgene or OriGene. We have now included detailed information on plasmid construction, source, and sequence identifiers in the revised Materials and Methods section. Specifically, Addgene IDs, UniProt accession numbers, and cloning details are provided in Supplementary Table S2C to ensure full transparency and reproducibility.

8. In line 617, "penicillin/streptomycin" is mentioned redundantly and should be corrected.

We thank the reviewer for pointing that out and this redundancy is now corrected in the new version of the manuscript.

Reviewer #2 (Remarks to the Author):

Summary

This manuscript describes a library for engineering synthetic promoters that are responsive to transcription factors associated with T cell activation and exhaustion. The authors combine minimal promoters with repetitive TFBSs to demonstrate promoter activity in HEK293 and Jurkat cells, and integrate these promoters into Boolean circuits

and noise-reducing motifs. Finally, the NR4A2-responsive SP was validated in primary human T cells. However, the design strategy seems closely related to techniques widely used in existing research and lacks direct comparisons with established synthetic promoters. Moreover, the deep learning model's contribution is not fully substantiated, with its performance metrics and integration with experimental data remaining inadequately demonstrated. Collectively, these issues diminish the overall innovation and significance of the work.

We thank the reviewer for the thoughtful assessment of the strengths and limitations of our work. In response to this feedback, we have revised the manuscript to improve clarity, add supporting analyses where appropriate, and better articulate the scope and intent of the study. We would like to clarify that the goal of this work is not to introduce a fundamentally new promoter design methodology, but rather to develop and systematically characterize a library of synthetic promoters using binding sites that respond to transcription factors associated with T cell activation and persistent stimulation. While the modular use of minimal promoters and repetitive TF binding sites has precedent in the field, our contribution lies in the scale, systematic evaluation, and application-oriented integration of these elements into a coherent toolkit suitable for use in T cells. Regarding comparisons with existing synthetic promoters, our intention was not to perform a comprehensive benchmarking study, but to contextualize the behavior of selected SPs relative to previously reported designs where appropriate. We have clarified this positioning in the revised manuscript to avoid implying direct replacement or superiority over established systems.

Major comments

1.The synthetic promoters constructed using repetitive TFBSs have been extensively reported in previous studies (e.g., Cell, 2017, 171(5): 1138-1150. e15; Nat. Commun., 2019, 10(1): 2880). Recent work (Nat. Commun., 2024, 15(1): 10353) has demonstrated the construction of thousands of synthetic promoters by combining three types of minimal promoters with various repetitive TFBS sequences characterized in HEK293 cells, which is the same cell line used in this study.

The manuscript should more prominently emphasize its innovative aspects and key highlights. Moreover, a direct comparison with existing synthetic promoter systems (e.g., ACS Synth. Biol., 2022, 11(4): 1440-1453; Science, 2022, 378(6625): 1227-1234) is needed to clearly demonstrate the advantages of the new design.

Our answer is included in the comment below.

2.The authors demonstrated the activation of the NR4A2-responsive SP in primary human T cells, highlighting its potential for practical applications. However, a direct comparison with existing synthetic promoters (e.g., NFAT and NR4A-based promoters reported in ACS Synth. Biol., 2022, 11(4): 1440–1453) is necessary to clearly showcase the improvements offered by the new design. Without such comparative data, the purported improvements remain unsubstantiated.

We thank the reviewer for raising these important points and for highlighting several foundational studies in synthetic promoter design. While we agree that prior works, such as Nissim et al. (Cell, 2017), Wu et al. (Nat. Commun., 2019), and Zahm et al. (Nat. Commun., 2024) among others (e.g. Stanton et al ACS Synthetic biology 2015 which we have contributed to), have advanced the development of synthetic promoters (SPs) using repetitive TF binding sites (TFBSs), we introduce novel synthetic promoters with binding sites that were not previously reported.

In the remarkable works from Nissim et al (2017) and Wu et al (2019), the synthetic promoters were designed to respond to TF upregulated in (i) cancer cells vs normal cells, (ii) cancer cells from cancer stem cells or in (iii) in vitro organoid differentiation model. Finally, the important work from Zahm et al. (2024) reported a large MPRA-based screen of 6,000+ SPs in HEK293 cells. Considering these work all together, promoters responding to some transcription factors we tested (e.g. IRF4, IKZF2, AF motifs with TCA cores, NR4A2 consensus elements), specific BS, or minimal promoters and architectures differ from our research. Finally, we thank the reviewer to point to this other important work on the design of synthetic zinc finger transcription regulators and cognate responsive promoters developed by the Khalil group. While we acknowledge the remarkable design and test also in CAR-T cells, we would also clarify that we did not design new synthetic TF/promoters pairs but rather synthetic promoters that respond to endogenous transcription factors. Therefore, our system is not directly comparable to the above mentioned one.

Finally, with specific reference to Guo et al 2022-existing activation-responsive promoters such as NFAT- or NR4A-based systems- these represent well-established approaches for sensing acute TCR signaling. Our work is not intended to benchmark or outperform these promoters; rather, we expand the available design space by providing a collection of synthetic promoters responsive to multiple TFs linked to activation and chronic stimulation, offering a range of transcriptional behaviors that can be selected according to experimental or application-specific needs.

However, as suggested by the reviewer, we have carried out a comparison with an NR4A promoter previously described by Guo et al. (2022), since NR4A2 is included among the transcription factors targeted in our library. A direct comparison between NFAT- and NR4A-based promoters has already been reported in that study, and therefore we did not repeat this analysis here.

In this experimental setting, SP73 exhibited higher inducible activity than the previously reported NR4A promoter in both HEK293 and Jurkat cells (**New Supplementary Figure S24A-B**). However, we would like to underscore that this comparison is intended to place SP73 in the context of existing NR4A-responsive designs rather than to establish a general benchmark, since the goal of our study is not to identify a single promoter that universally outperforms previously reported systems, but to provide a set of synthetic promoters spanning an operational range of transcriptional behaviors. This enables the selection of an SP with properties that best match the requirements of a given downstream application.

Given the importance of all the references pointed out by the reviewer, we have included the missing ones in the revised version of the manuscript.

3. Several critical details are missing regarding the deep learning model, such as the source of the ChIP-seq dataset, the composition of the validation set, and overall model performance. Additionally, applying the pre-trained model directly to a small dataset

(~80 sequences) without fine-tuning is problematic, as indicated by the modest correlation ($r = 0.49$). This performance makes it difficult to extract reliable and useful insights from the model.

We thank the reviewer for this thoughtful comment. We agree that the modest correlation between predicted and experimentally measured promoter rankings does not support strong claims of model generalizability between the external ChIP-seq dataset and the T-cell promoter library used in this study.

The purpose of including the transfer learning analysis was not to derive biological conclusions that drive subsequent experiments, but rather to explore whether promoter ranking could be partially inferred from externally trained models in situations where the dataset of interest is too small to support de novo model training. In many synthetic biology applications, promoter libraries are limited in size, making traditional supervised machine learning approaches impractical. We therefore evaluated whether a pre-trained model could capture relative trends in promoter strength without fine-tuning.

The ChIP-seq dataset used for model training and internal validation was obtained from the Eukaryotic Promoter Database, where curated datasets have been assembled from published studies. The ~80-sequence T-cell promoter dataset was treated strictly as an independent test set, and no fine-tuning was performed using these data. We have clarified this training-validation-test schema in the revised manuscript to ensure transparency.

We acknowledge that the predictive performance is moderate and does not substitute for experimental characterization. Accordingly, we present this analysis as exploratory and methodological rather than as a central claim of predictive capability. The core conclusions of the study remain fully supported by experimental data independent of the transfer learning analysis.

4. Promoter activation is evaluated by co-expressing the corresponding transcription factors with the synthetic promoters. Given that promoter activity is dose-dependent and that variations in coding sequences can lead to differences in protein expression levels in mammalian cells, the resulting TF concentrations may vary significantly among constructs. Consequently, absolute induction values across different TF types may not be directly comparable. It is essential to quantify and normalize TF expression levels to ensure a meaningful functional comparison.

We agree with the reviewer that promoter activation is assessed by co-expression of the synthetic promoter (SP) with the corresponding transcription factor (TF), and that promoter activity is inherently dose-dependent on TF abundance.

For each TF-responsive group, the same TF expression construct was used across all conditions, thereby minimizing coding-sequence-dependent variability within each TF–SP set. In addition, all SPs drive expression of the same fluorescent reporter, limiting variability related to reporter stability or translation efficiency.

We acknowledge that TF expression levels may differ between constructs and across cell types. Accordingly, our experimental design was not intended to enable direct quantitative comparisons of absolute induction values across different TF types. Rather, our analysis focuses

on intra-TF comparisons, allowing us to evaluate relative promoter properties such as OFF-state basal activity and inducibility within each TF-responsive group.

To directly address the reviewer's concern, we quantified TF transcript levels following transient delivery in HEK293 and Jurkat cells (**new Supplementary Figure S35**). qPCR analysis revealed substantially lower TF mRNA levels—approximately two to three orders of magnitude—in Jurkat cells compared to HEK293 cells. While mRNA quantification does not directly measure protein abundance, the magnitude of transcript reduction provides a mechanistic explanation for the reduced activation amplitude observed in Jurkat cells.

Importantly, despite differences in activation magnitude between cell types, the relative ranking of promoter performance was largely preserved across delivery systems and cellular contexts. This supports the conclusion that reduced TF availability, rather than loss of promoter specificity or robustness, accounts for the lower absolute induction observed in Jurkat cells.

We have clarified these points in the revised manuscript to ensure that the scope and interpretation of promoter comparisons are appropriately defined.

5. The term “noise” is frequently mentioned but is not clearly defined or quantified, particularly with respect to the OFF state of the promoters. A detailed explanation of how noise is calculated, along with its biological significance, is necessary. Moreover, the limited number of experimental replicates (often only two) raises concerns about the statistical robustness of these measurements; additional replicates or more rigorous statistical analysis would improve confidence in the reported values.

We thank the reviewer for raising this important point. In this study, we use the terms *noise* or *leakiness* to refer specifically to basal promoter activity in the absence of the cognate transcription factor (TF), i.e., reporter expression measured in the OFF state. Operationally, OFF-state activity was quantified as the geometric mean fluorescence intensity (gMFI) of mCherry within the transfected (EGFP-positive) cell population under no-TF conditions (e.g., **Figure 1**). Thus, the term does not refer to stochastic transcriptional variability at the single-cell level, but rather to unwanted basal activation of the synthetic promoter.

Biologically, minimizing OFF-state activity is particularly relevant when synthetic promoters are coupled to potent effector or therapeutic payloads, where unintended basal expression could result in off-target effects or toxicity. Accordingly, our analysis focuses on comparative assessment of basal activity across promoter architectures and minimal promoters to identify configurations with improved ON/OFF dynamic range.

To improve clarity, we have now explicitly defined OFF-state activity in the revised manuscript and detailed its calculation in the figure legends and Materials and Methods section. We have also included a representative flow cytometry dot plot illustrating OFF-state activity (**new Supplementary Figure S5C**).

Regarding statistical robustness, we acknowledge that some initial experiments were performed with $n=2$ independent biological replicates. In response to the reviewer's concern, we have conducted additional independent experiments to increase replication to at least $n=3$ in both HEK293 and Jurkat cells, for the relevant datasets in which the initial tests identified promising SPs in HEK293 cells. The corresponding figures have been updated accordingly, and statistical analyses have been revised to reflect the expanded sample size.

Minor comments

1. While the manuscript analyzes transcription factors upregulated during various stages of T cell activation and exhaustion (Supplementary Figs. 1 and 2), a more precise quantitative assessment of TF expression levels would further justify the selection criteria used.

We thank the reviewer for this comment. The selection of transcription factors (TFs) was guided by a quantitative analysis of previously published RNA-seq datasets, rather than by qualitative inspection alone. As described in the *Materials and Methods* section, we analyzed multiple independent transcriptomic studies comparing naïve, activated, and chronically stimulated/exhausted T cells, and TFs were selected based on their consistent upregulation and absolute expression levels across these datasets.

In Supplementary Figures 1 and 2, TF expression is displayed as $\log_2$ TPM (transcripts per million) values to provide a direct representation of transcript abundance across conditions. This choice was intentional, as absolute expression levels are particularly relevant for synthetic promoter design, where TF abundance can directly impact promoter responsiveness. In this context, TFs were prioritized not only for differential expression, but also for being expressed at sufficiently high levels to support functional sensing.

To further clarify this point and facilitate interpretation, we have added a quantitative summary of TF expression values derived from the RNA-seq analyses in a new version of Supplementary Table S1, and we have clarified in the figure captions that the heatmaps represent expression levels rather than fold changes. We believe this provides a transparent and quantitative justification for the TF selection criteria used in this study.

2. In Figure 2B, the no-TF control for the IRF4/BATF group (e.g., SP61-No TF) shows unexpectedly high basal expression, contradicting the narrative in the text. This inconsistency should be thoroughly investigated and clarified.

We thank the reviewer for pointing out this inconsistency. The description in the text was based on the overall trend observed across experiments and was primarily informed by the results obtained in HEK293 cells. Upon re-examining the Jurkat cell data in response to the reviewer's comment, we identified that one experimental repetition showed elevated basal activity for the IRF4/BATF no-TF control (e.g., SP61-No TF), which contributed to the representation in Figure 2B. To clarify this point, we performed additional independent experiments in Jurkat cells. These additional measurements showed lower basal activity for the IRF4/BATF no-TF condition, consistent with the general interpretation presented in the manuscript. The figure has been updated accordingly in the revised version to reflect these results more accurately. We have also revised the text to avoid overgeneralization and to ensure that the description of basal activity is fully consistent with the updated data.

3. The manuscript contains redundant statements; for instance, lines 266–268 repeat the process of applying the pre-trained network to rank the synthetic promoters based on reporter fluorescence.

We thank the reviewer to point that out. We have now corrected this redundancy in the updated version of the manuscript.

4. There are duplicate references—for example, the Wu et al. Nat. Commun. 10, 717 (2019) citation appears twice.

We thank the reviewer for pointing that out. We have now corrected this redundancy in the updated version of the manuscript.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4 (Remarks to the Author):

Manuscript of Bonfa and Siciliano titled "Design of novel synthetic promoters to tune gene expression in T cells."

The study aims to develop the control of transgene expression in cells. Specifically, Chimeric-Antigen-Receptor T cells (CART) are of great clinical interest nowadays, utilizing constitutive strong promoters for transgene expression. Authors envisioned a most interesting change of the method by screening a library of some 80 synthetic promoters that are suggested to respond to seven major transcription factors of activated or exhausted human T cells, namely: IRF4, BATF, MAF, GATA3, NR4A2, EOMES, IKZF2 (also known as IKAROS). The list of TFs was selected from extensive datasets, and filtration according to RNA-seq data, which is already interesting. Notably, some are well known, and some are less considered of CART so far. Known TF-binding sites were cloned as 5-tandem repeats with a minimal promoter (selected from YB-TATA, miniCMV, miniTK, or LateADEp).

Cloned promoters were tested in HEK293 and Jurkat cell lines. That a reasonable start—but must recognize that HEK are not T-cells, and Jurkat cell lines (and subclones) were reported to have impaired signaling. This is perhaps the major limitation of this study. One other limitation to consider is the use of plasmids. CART are currently made with Retro- or Lenti-vectors that integrate into the genome. Using plasmids is legitimate, yet it might differ from viral vectors (it gains a transient expression, not permanent integration as CART is currently being manufactured).

Data showing the expression from synthetic promoters w/o stimulation (authors call it "Background noise" or "leakiness"). Induction of selected promoters with the TFs is brilliantly demonstrated in Figure 2, with the notion of limitation stated above. Authors smartly moved on to design and test more sophisticated synthetic promoters that encompass multiple TFs response elements that are designed for boolean logic gates—as shown in figure 3, each synthetic-promoter drives split-GAL4VP16 transcription

factor. This ambitious part is truly remarkable, yet we must also recognize possible limitations- as the biological activity of complicated promoters do not always follow strict logical roles (unfortunately). Authors go further, using the rtTA-system to allow additional external control (as published by several studies), demonstrating how their novel promoter may work together with the rtTA-DOX system to control the expression w/o the TFs. Finally, primary human CD8 are shown to work with a selected promoter and elevate reporter-gene expression at chronic-stimulation state in vitro; HEK and Jurkat cell lines were shown to secrete IL12 as an example for an interesting effector rather than reporter-gene only.

Overall, the study is very interesting and will be published.

We thank the reviewer for the careful reading of the manuscript and for the balanced and constructive assessment of both its strengths and limitations. We appreciate the recognition of the conceptual motivation behind this work. We agree that the use of HEK293 and Jurkat cell lines represents an initial and simplified experimental framework. HEK293 cells were intentionally employed as a reductionist system to validate promoter responsiveness and architecture in a well-controlled context, while Jurkat cells were used as a commonly adopted T cell model to assess promoter behavior in a lymphoid background. As noted by the reviewer, Jurkat cells exhibit known signaling alterations, and we therefore avoided over-interpreting these data. Importantly, we extended our analysis to primary human CD8⁺ T cells, where we demonstrate that a selected synthetic promoter responds to persistent stimulation in vitro by NR4A2 upregulation, supporting the relevance of the approach in a more physiologically meaningful setting.

We also acknowledge the reviewer's point regarding the use of plasmid-based expression systems. Plasmids were chosen to enable rapid and systematic screening of a large promoter library and circuit architectures. While episomal expression may differ from genomic integration achieved by retroviral or lentiviral vectors, this approach allowed us to focus on promoter-intrinsic properties such as basal activity, inducibility, and dynamic range, and to identify promising promoters for genomic integration such as the ones responding to NR4A2. With respect to the implementation of multi-input promoters and Boolean logic architectures, we appreciate the reviewer's acknowledgement of both the ambition and the inherent biological complexity of such designs. Our intent was not to claim strict digital behavior in all contexts, but rather to demonstrate that these synthetic promoters are compatible with circuit-level integration and can be combined to achieve more selective transcriptional control. We have taken care to present these results as proof-of-concept demonstrations and to avoid overgeneralization.

Finally, we thank the reviewer for recognizing the overall contribution of this work. We have revised the manuscript to better emphasize its scope as a platform-oriented and exploratory study, and to clearly delineate current limitations and future directions.

Major comments:

1. Authors must clearly declare limitations, including the above-mentioned work with cell lines and non-integrating plasmids. In the last part, they did use a lentivector for the

primary human T cells, perhaps mentioning the correlation between plasmids and LVs if many of these were co-studied.

We thank the reviewer for highlighting the importance of clearly stating the limitations of this study. We agree that the use of immortalized cell lines and non-integrating plasmid-based expression systems represents an important consideration that should be explicitly acknowledged.

In this work, HEK293 and Jurkat cells were intentionally used as reductionist and scalable model systems to enable systematic screening and characterization of a relatively large library of synthetic promoters. As noted by the reviewer, HEK293 cells are not T cells, and Jurkat cells are known to exhibit altered signaling pathways. In addition, we found that electroporation in Jurkat cells was not sufficiently reliable for large-scale screening purposes. For these reasons, we limited the interpretation of results obtained in these cell lines to comparative and exploratory analyses and avoided extrapolating these findings directly to therapeutic settings.

Given the limited availability of buffy coats for the isolation of primary human T cells, we focused our validation efforts in primary CD8⁺ T cells on the NR4A2-responsive synthetic promoter, which showed the most robust and consistent behavior in both HEK293 and Jurkat cells. We agree that broader validation directly in primary T cells, ideally using integrated vectors, will be important in future studies.

We also acknowledge that the majority of promoter screening experiments were performed using transient plasmid transfection. This approach was chosen to facilitate rapid iteration and side-by-side comparison of promoter designs. We agree that episomal expression may differ from genomic integration achieved by retroviral or lentiviral vectors commonly used in CAR T cell manufacturing.

Importantly, to address translational relevance, we validated one of the best-performing synthetic promoters using a lentiviral vector in primary human CD8⁺ T cells, where promoter activity was observed under conditions of persistent stimulation. While we did not perform a systematic head-to-head comparison between plasmid-based and lentiviral delivery for all promoters, the consistency of promoter activation observed across these contexts suggests that the core TF-responsive behavior is retained. We have clarified in the revised manuscript that this observation is qualitative and that a comprehensive comparison between episomal and integrated systems remains an important direction for future work.

We have now revised the Discussion to explicitly state these limitations and to clearly delineate the scope of the current study as a platform-oriented and exploratory investigation.

Moreover, following reviewers' suggestions, we have taken initial steps in this direction by evaluating the best-performing NR4A2-responsive promoter (SP73) in an adoptive T cell transfer setting. In collaboration with Professor Luca Gattinoni's group (Leibniz Institute for Immunotherapy, University of Regensburg, Germany), we assessed SP73 activity in NY-ESO-1-specific CD8⁺ T cells in a humanized melanoma xenograft model. Tumor-infiltrating T cells exhibited detectable SP73-driven reporter expression, indicating that the promoter can be activated in vivo within the tumor microenvironment. Importantly, reporter activity

dynamically decreased following tumor regression, consistent with reduced NR4A2 levels and supporting the reversible and state-dependent behavior observed in vitro.

Collectively, these observations support the translational potential of the SP platform presented here and suggest that SP73 can function as an in vivo sensor of NR4A2-driven transcriptional activity. We view these results as encouraging preliminary evidence and a strong motivation for future studies aimed at systematic in vivo evaluation and therapeutic implementation. The data are included in the revised manuscript as new **Figures 6-7**.

2. Supplementary data is exceptionally good- consider moving representative raw data (aka FACS plots) of unstimulated and stimulated cells to maintext- so readers can appreciate the quality of your data!

We thank the reviewer for this very positive comment and for the helpful suggestion. Given the new data generated, we could only move few other plots around. We have added the orthogonality matrix that show the specificity of SP to cognate TF, and for the in vitro and in vivo characterization of promoters responding to NR4A2 in primary T cells we have also added FACS plot.

3. This study is focused on the design of novel synthetic promoters for CART. Authors better refer to previous studies that aimed at the same target- and let the reader appreciate the novelty of their approach (which is truly of interest for the broad readership of Nature Communication).

We thank the reviewer for this constructive suggestion. We agree that placing our work more explicitly in the context of prior studies aiming to control transgene expression in CAR T cells is important to help readers fully appreciate the novelty and scope of our approach.

Accordingly, we have expanded the Introduction and Discussion to more clearly reference previous efforts that have employed constitutive or inducible promoters, as well as signal-responsive transcriptional systems, for CAR T cell engineering. These include studies using NFAT-, or cytokine-inducible promoters, as well as synthetic circuit-based approaches designed to couple antigen recognition to payload expression⁴⁻⁸.

In doing so, we clarify that the novelty of our work does not lie in introducing an entirely new class of promoter architectures, but rather in the systematic design and characterization of a curated library of TF-responsive synthetic promoters specifically motivated by transcriptional programs associated with T cell activation and persistent stimulation. Unlike prior approaches that typically rely on one or a few canonical sensors, our study explores a broader set of transcription factors, evaluates promoter-intrinsic properties such as basal activity and inducibility, and demonstrates compatibility with circuit-level integration and external control systems.

We believe that this contextualization more clearly positions our work as a platform-oriented and enabling contribution to CAR T cell synthetic biology, and we have revised the manuscript accordingly to guide readers in understanding how our approach complements and extends existing strategies.

4. Titles of figures captions are somewhat descriptive of intention, data is very good- and authors are encouraged to let some more active caption-titles that highlight their achievements.

We thank the reviewer for this encouraging comment. We agree that clearer and more informative figure caption titles can help guide readers and better highlight the key findings, especially given the quality and breadth of the data. Captions have been revised and included in the new manuscript.

5. The last part, demonstrating the activity of the synthetic promoter in primary human T cells following chronic stimulation in vitro, is referred to as "Functionality in primary human T cells upon antigen stimulation" (lines 100-101). However, as far as I can see, these T cells were stimulated using the strong anti-CD3+anti-CD28 beads, not by specific antigens. Please excuse me if I missed something (perhaps data not shown, or my limited understanding); alternatively do change the text to fit with the presented data, which is a strong activation NOT of a specific antigen.

We thank the reviewer for carefully pointing this out and for the opportunity to clarify the terminology used. The reviewer is correct that, in the experiments shown, primary human CD8⁺ T cells were stimulated using anti-CD3/anti-CD28 beads, which provide a strong and non-antigen-specific activation signal, rather than stimulation through a defined cognate antigen. In the revised manuscript we also expand the in vitro characterization of NR4A2-responsive synthetic promoters by analyzing six SP variants under repeated anti-CD3/anti-CD28 stimulation, including assessment of promoter activation during persistent stimulation and reversibility following bead withdrawal and re-stimulation.

In addition, to address antigen-specific activation directly, we performed a separate set of experiments in which primary human CD8⁺ T cells were engineered with a T cell receptor (TCR) specific for the NY-ESO-1 antigen together with the NR4A2-responsive promoter SP73. In this setting, we demonstrate activation of the synthetic promoter upon co-culture with NY-ESO-1-expressing SK-MEL-23 melanoma cells. Furthermore, we show promoter activation in a humanized mouse model following subcutaneous tumor implantation and adoptive transfer of the engineered T cells (new Figures 5-7).

Minor comments:

1. Why did you choose 5Xrepeats?

We chose to use five tandem repeats of each transcription factor binding site (TF-BS) as a design compromise informed by prior synthetic promoter studies and by practical considerations for promoter screening.

Previous work has shown that multimerization of TF-BSs is generally required to achieve measurable and robust inducible activity from minimal promoters, while excessive repetition can increase basal activity, introduce context-dependent effects, or reduce portability across cell types. In this context, five repeats represent a commonly used intermediate configuration that is sufficient to support inducibility while limiting promoter length and minimizing OFF-state activity.

Importantly, our goal was not to optimize the number of TF-BS repeats for each individual transcription factor, but rather to adopt a uniform design that would enable systematic comparison across multiple TFs and promoter architectures. Using a fixed number of repeats allowed us to focus on relative differences in basal activity, inducibility, and compatibility with circuit-level designs, without introducing additional variables related to repeat number.

We note that alternative configurations (fewer or more repeats) may be advantageous for specific TFs or applications, and exploring this design space represents an important direction for future work.

2. How do you envision utilizing such a transgenic-TFs system in vivo? If rather than let it go with lentivectors and dependence on endogenous TFs, please consider and write expected differences between T-cell subtypes.

We thank the reviewer for this important question. In vivo, we do not envision transgenic transcription factor (TF) overexpression as the intended operational mode of this system. In the present study, TF co-expression was used as a screening and validation perturbation to confirm cognate promoter responsiveness and to enable controlled comparison of synthetic promoter architectures.

For in vivo applications, as shown in the revised manuscript, the selected synthetic promoter is delivered to primary T cells using lentiviral integration, together with a tumor-targeting receptor (TCR or CAR, depending on the application), and is designed to respond to endogenous TF activity. This approach is consistent with current CAR T cell manufacturing practices and avoids potential safety and functional concerns associated with constitutive TF overexpression.

As correctly noted by the reviewer, differences in promoter behavior are expected across T cell subsets due to distinct activation kinetics and transcriptional programs. For example, CD8⁺ and CD4⁺ T cells differ in their differentiation trajectories and TF usage; transcriptional programs associated with cytotoxic function and persistent stimulation are often more prominent in CD8⁺ T cells, whereas CD4⁺ subsets can engage alternative TF networks depending on lineage and activation state. In addition, naïve, effector, memory, and regulatory T cell populations are expected to exhibit different baseline and inducible TF activities, which may influence both OFF-state activity and inducibility of TF-responsive synthetic promoters.

In the present work, primary CD8⁺ T cells were specifically isolated from PBMCs to reduce heterogeneity and to focus on a subset most directly relevant for cytotoxic and CAR T cell applications. We acknowledge that systematic evaluation across additional T cell subsets will be important for future studies aimed at subtype-specific calibration and broader translational deployment.

3. If you have data of multiple promoters tested as plasmids and as lentivectors, this might be of interest to the broader readership, as your method is not limited to CART.

We thank the reviewer for this insightful suggestion. We agree that comparing promoter behaviour across transient and integrating expression systems is of interest beyond CAR T cell applications.

In the course of this study, we tested six variants of NR4A2-responsive synthetic promoters both by transient plasmid expression in cell lines (Supplementary Figures S11 and S17) and by lentiviral transduction in primary human CD8⁺ T cells (new **Figure 5**). This provided an

opportunity to qualitatively compare promoter performance across episomal and integrated contexts. Across these experimental settings, we consistently observed that SP73 exhibited the strongest inducible response, followed by SP78 and SP75.

While these experiments do not constitute a systematic or quantitative comparison between plasmid-based and lentiviral delivery for the full promoter library, the concordant ranking observed for these NR4A2-responsive promoters suggests that their relative performance is preserved across expression modalities. We have added this observation to the Discussion to highlight the broader applicability of the approach, while clearly noting that more extensive comparisons across additional promoters and cell types will be required in future studies.

REFERENCES

1. Niu, H. & Wang, H. TOX regulates T lymphocytes differentiation and its function in tumor. *Front. Immunol.* **14**, (2023).
2. Mader, S. & White, J. H. A steroid-inducible promoter for the controlled overexpression of cloned genes in eukaryotic cells. *Proceedings of the National Academy of Sciences* **90**, 5603–5607 (1993).
3. Nissim, L. *et al.* Synthetic RNA-Based Immunomodulatory Gene Circuits for Cancer Immunotherapy. *Cell* **171**, 1138–1150.e15 (2017).
4. Greenshpan, Y. *et al.* Synthetic promoters to induce immune-effectors into the tumor microenvironment. *Commun. Biol.* **4**, 143 (2021).
5. Hooijberg, E., Bakker, A. Q., Ruizendaal, J. J. & Spits, H. NFAT-controlled expression of GFP permits visualization and isolation of antigen-stimulated primary human T cells. *Blood* **96**, 459–66 (2000).
6. Fischer, A. *et al.* Cytokine-inducible promoters to drive dynamic transgene expression: The “Smart Graft” strategy. *Xenotransplantation* **27**, (2020).
7. Roybal, K. T. *et al.* Engineering T Cells with Customized Therapeutic Response Programs Using Synthetic Notch Receptors. *Cell* **167**, 419–432.e16 (2016).
8. Ilaria, D. M., Luigi, R., Matteo, M. & Velia, S. Engineering a PD-L1–sensing synthetic receptor for programmable macrophage-mediated phagocytosis. Preprint at <https://doi.org/10.64898/2026.01.21.700810> (2026).

We are grateful to the reviewers for their feedback and support. We have carefully addressed outstanding points raised by Reviewer #1 and performed substantial additional cloning and experiments where appropriate. Below, we provide a detailed point-by-point response, with corresponding revisions incorporated into the manuscript.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Bonfá et al. have addressed several of the previously raised issues, including the addition of in vivo validation for the NR4A2 SP in a mouse xenograft model, and the revised Introduction better articulates the intended scope of the study. However, my major concerns remain unresolved, and in my view the revised manuscript still does not provide the level of conceptual advance, rigor, or physiological relevance expected for publication in Nature Communications.

Major point 1

Despite the revised title and Introduction, the manuscript still overstates the breadth and versatility of the proposed toolkit. The study is presented as a set of synthetic promoters responsive to multiple TFs associated with activation and chronic stimulation, offering a spectrum of transcriptional behaviors for distinct applications. However, this claim is not supported by the actual design of the study.

All seven TFs used for SP design are derived from a single T-cell state, namely exhausted or chronically stimulated T cells.

This immediately limits the generality of the proposed toolkit. TF activity differs substantially across naïve, effector, memory, and exhausted T-cell states, and these states are governed by distinct regulatory networks. A genuinely versatile promoter toolkit would therefore require responsive elements linked to TFs active across multiple, biologically distinct T-cell states. In its current form, the manuscript does not establish such breadth.

More importantly, only the NR4A2 SPs were evaluated under endogenous TF regulation. The SPs responsive to the other six TFs were tested only in the context of exogenous TF overexpression. Their behavior within native T-cell regulatory circuits therefore remains entirely uncharacterized. This is not a minor gap; it directly undermines the manuscript's core claim that these promoters can harness endogenous TF dynamics for programmable control in T cells.

We thank the reviewer for this observation. To directly address the concern regarding endogenous TF regulation, we performed additional experiments in primary human CD8⁺ T cells isolated from three independent donors. We selected representative synthetic promoters that exhibited inducible activity in HEK293 and Jurkat cells and introduced them into primary T cells by nucleofection. Cells were subjected to two rounds of CD3/CD28 stimulation before nucleofection and one round post-nucleofection and both endogenous transcription factor expression and promoter-driven mCherry expression were quantified 48 h after nucleofection (new Figure 5A-B; Supplementary Figure S42, Supplementary Figure S43). Despite the relatively limited transfection efficiency (~ 20-30%) inherent to primary T-cell nucleofection, promoter activation was detected for MAF-, IRF4/BATF-, EOMES-, and NR4A2-responsive constructs. By contrast, GATA3- and IKZF2-responsive promoters showed minimal activation, consistent with our observations in HEK293 and Jurkat cells (new Figure 5A).

These experiments directly address the reviewer's concern regarding endogenous TF regulation and demonstrate that representative synthetic promoters from five of the seven TF-responsive groups tested are activated by endogenous transcriptional programs in primary human T cells, thereby extending endogenous validation beyond the NR4A2-responsive subset.

To further address the reviewer's concerns regarding the scope of the study, we revised the Introduction, Results, and Discussion to more clearly state that the objective of this work is the systematic development and characterization of TF-responsive synthetic promoters that sense endogenous transcription factor activity during antigen stimulation, rather than the classification of discrete T-cell functional states. Accordingly, we have refined the wording throughout the manuscript to avoid overinterpretation of the breadth of the toolkit and to better align the claims with the experimental evidence presented.

Major point 2

The authors repeatedly state in the response letter that their goal is neither to benchmark against prior NR4A-based systems nor to develop exhaustion-specific SPs, but rather to establish a modular SP toolkit. However, the work ultimately does not deliver a substantively new use case beyond antigen-responsive NR4A SP activation.

That application has already been reported and is conceptually very similar to prior NR4A-based systems, as well as to more established NFAT-based activation-responsive promoters. This limited advance is a serious problem for a journal at this level. Although the manuscript includes screening of numerous SPs, the downstream validation converges almost entirely on a single promoter class that is already comparatively well

developed in the literature. As a result, the study does not convincingly establish either a broadly enabling platform

We thank the reviewer for this comment. We respectfully disagree that the contribution of the study ultimately converges to a previously described NR4A-responsive use case.

The primary objective of the work is not the validation of a single NR4A-responsive promoter, but the design and characterization of a library of transcription factor-responsive synthetic promoters spanning multiple transcriptional inputs, exhibiting distinct activation amplitudes, OFF-state activities, fold inductions, orthogonality profiles, and compatibility with higher-order circuit architectures. In this regard, the principal contribution of the manuscript is the generation of a modular collection of TF-responsive elements together with the associated design principles, rather than a single downstream application.

The NR4A2-responsive subset was selected for deeper validation because it emerged as the most robust candidate during screening.

However, even within this subset, the study goes substantially beyond previous reports.

Compared with prior NR4A-based systems, we characterize multiple promoter architectures with distinct activation strengths, demonstrate dynamic ON–OFF and re-inducible behavior in primary human T cells, and provide the first in vivo demonstration of a NR4A2-responsive synthetic promoter in engineered T cells.

These features were not investigated in previous NR4A-responsive systems. In this respect, we note that Reviewer #4 reached a different conclusion regarding the conceptual contribution of the work, highlighting that the study provides original data and a systematically characterized set of synthetic promoters, and that the existence of related promoter systems in the literature does not diminish the value of introducing new tools and design principles. In this perspective, our goal is not to replace previously reported NR4A- or NFAT-based systems, but to expand the available repertoire of TF-responsive promoter architectures and provide a framework for their systematic evaluation.

Finally, while the present study focuses on T-cell biology, the selected transcription factors are not restricted to T cells. Factors such as NR4A2, EOMES, IRF4, BATF, and c-MAF play important roles in neuronal, developmental, and oncogenic transcriptional programs. Consequently, the promoter architectures described here may be adapted in future studies to sense and regulate transcriptional activity in biological contexts beyond engineered T cells. We do not claim such applications in the present manuscript; however, they further illustrate that the conceptual contribution of the work extends beyond the specific NR4A2 validation presented here.

Major point 3

I appreciate that the authors quantified TF mRNA levels in Jurkat cells to assess electroporation performance. However, I do not agree with the conclusion that “while activation amplitudes differed between cell types, consistent with reduced TF availability, the relative behavior of many promoters was conserved.”

It therefore remains unclear whether these differences reflect poor transfection efficiency, broader technical variability, or genuine cell-type-specific regulatory effects. This uncertainty is because it directly affects interpretation of promoter behavior in the more relevant T-cell context. A more rigorous strategy would have been to incorporate a fluorescent reporter such as BFP into the TF-expression plasmids, analogous to the dual-promoter constructs used in primary T cells, and then gate on BFP-positive cells,

We thank the reviewer for this important technical suggestion. We agree that gating promoter output on cells that have effectively received and expressed the TF construct provides a more rigorous assessment of promoter activity in Jurkat cells, particularly given the variability associated with electroporation.

To directly address this point, we generated TF-2A-GFP (for all TFs except MAF, for which cloning was not successful due to construct-related technical issues) allowing TF-delivered cells to be identified by flow cytometry. For BATF, we used BFP instead of GFP to enable simultaneous analysis in the IRF4/BATF setting.

We then repeated the Jurkat electroporation experiments and quantified synthetic promoter-driven mCherry expression separately within fluorescent reporter-positive and reporter-negative populations (accounting for both GFP and BFP in the IRF4/BATF coexpression) (New Supplementary Figure S41). This analysis directly addresses the concern that low or heterogeneous TF delivery may confound interpretation of promoter activity. In all tested cases, mCherry reporter activation was enriched within the TF-reporter-positive population, whereas TF-reporter-negative cells showed little to no mCherry induction (New Supplementary Figure S41). Thus, promoter activation correlated with successful TF construct expression at the single-cell level, despite limited nucleofection efficiency. These results support the conclusion that the observed promoter responses in Jurkat cells are dependent on TF delivery and are not simply a consequence of nonspecific electroporation variability or background reporter activation.

Importantly, this experiment also helps explain why promoter activation amplitudes in Jurkat cells were generally lower than in HEK293 cells: only the fraction of Jurkat cells that successfully received and expressed the TF construct contributed to the inducible response. By gating directly on TF-reporter-positive cells, we were able to more accurately assess promoter responsiveness in the relevant population.

We have added these new data to the revised manuscript and updated the corresponding Results and Methods sections. We have also revised the discussion of the Jurkat screening data to clarify that transient electroporation introduces technical variability, but that TF-gated analyses support the interpretation that promoter activation is linked to cognate TF expression.

1. Figure S2 and TF selection

The genes in Fig. S2 were clustered using Euclidean distance, but no dendrogram or equivalent visualization is provided. It is therefore difficult to assess whether the seven chosen TFs truly emerge from distinct clusters or whether they capture meaningfully different transcriptional behaviors during T-cell differentiation. This information would be important for understanding the logic of TF selection and for guiding future use of the proposed SPs.

We thank the reviewer for this observation. We would like to clarify that the Euclidean-distance clustering shown in Fig. S2 was included solely for visualization purposes and did not influence the selection of the seven TFs.

The orthogonality matrix is generally informative and supports the conclusion that cross-activation by non-cognate TFs is limited, with NR4A2 SPs appearing to perform best overall. However, GATA3 SPs are absent from Fig. 2K. In addition, because the manuscript is explicitly focused on T-cell applications, an orthogonality matrix generated in Jurkat cells would be more relevant and more convincing than one derived primarily from HEK293 cells.

We thank the reviewer for the careful evaluation of the orthogonality analysis and for these helpful suggestions.

Regarding the absence of GATA3-responsive SPs from Fig. 2K, we note that these promoters displayed low inducible activity under the tested conditions and therefore were not informative for comparative analysis of orthogonality among the best-performing TF-responsive promoters. To ensure completeness, we have now included the corresponding GATA3 orthogonality data in Supplementary Figure S36 and refer to them explicitly in the revised manuscript.

With respect to the use of HEK293 cells, we agree that T-cell-derived systems such as Jurkat cells are highly relevant for downstream applications. However, the purpose of the orthogonality matrix is distinct from the functional validation of promoter activity in a T-cell context. Specifically, the orthogonality analysis was designed to evaluate promoter–TF specificity and potential cross-activation between synthetic promoter modules and transcription factors under controlled conditions. In this setting, HEK293 cells provide a

more neutral background with high transfection efficiency and reduced interference from endogenous lineage-specific transcriptional programs, enabling a systematic assessment of intrinsic promoter–TF interactions.

We have clarified this rationale in the revised manuscript to better explain both the experimental design and the intended scope of the orthogonality analysis.

3. TOX-related SPs.

The fact that TOX is only weakly upregulated under persistent stimulation does not, by itself, argue against the possible utility of a TOX-responsive SP. One of the advantages of synthetic promoter design is that repeated core motifs can amplify transcriptional responses even when endogenous TF abundance is modest. It would therefore be helpful to indicate where TOX ranks within the transcriptomic datasets used for TF selection in Figures S1 and S2.

We thank the reviewer for this insightful comment and agree that low TF abundance alone does not necessarily preclude the utility of a synthetic promoter. Indeed, one advantage of synthetic promoter design is that multimerization of TF binding sites can amplify transcriptional responses even when endogenous TF levels are modest.

TOX was considered during the TF selection process and its expression levels are reported in the transcriptomic datasets used for prioritization. Specifically, TOX expression was 1.98 TPM in DICE, 0.57 TPM in Liblau, 19.6 TPM in June, and 1.62 TPM in Wherry datasets, whereas the highest-expressed TFs in the same datasets reached 2730.7, 4090.6, 2340.4, and 1028.6 TPM, respectively (Supplementary Table S1_new). Thus, although TOX has an established biological role in T-cell dysfunction, its absolute expression remained comparatively low across the datasets analyzed.

Importantly, we do not argue that a TOX-responsive promoter would necessarily be non-functional. Rather, given the large number of candidate TFs available for promoter design, we prioritized a subset of TFs for experimental validation.

We have clarified this point in the revised manuscript.

4. CCL21 quantification.

The issue with CCL21 quantification remains unresolved. If the authors cannot explain, or reproducibly confirm, why constitutive expression yields lower CCL21 output than SP73, then these data should be removed, as they currently risk misleading interpretation. Alternatively, the experiment should be repeated with a different therapeutic cargo for which expression can be quantified more robustly.

We thank the reviewer for this comment and agree that the behavior of the constitutive CCL21 control remains difficult to interpret. While we do not believe that this issue undermines the inducible activation observed for SP73 in HEK293 cells, we agree that

the current dataset may create ambiguity regarding the interpretation of CCL21 expression levels.

To avoid potential misinterpretation, we have removed the CCL21 data from the revised manuscript. Importantly, the proof-of-concept demonstration of inducible therapeutic payload expression remains supported by the IL12p70 experiments, which show robust and reproducible induction under SP73 control (Supplementary Figure S22).

We believe that removal of the CCL21 dataset provides a clearer presentation of the results and addresses the reviewer's concern.

5. SP91 preparation.

The preparation of SP91 is not described in the Methods or supplementary tables. This omission should be corrected.

We thank the reviewer for this careful observation. We have now added the missing information regarding SP91 preparation to the revised Materials and Methods section.

Briefly, SP91 was cloned upstream of the mCherry coding sequence using Mammalian Toolkit (MTK) assembly into the basic entry backbone pYTK_095 (Addgene #65202). This information is now included in the revised manuscript to ensure full reproducibility.

6. Chronic stimulation protocol.

The description of the chronic stimulation protocol using Dynabeads remains unclear. Specifically, the phrase "repeated stimulation every 48 h" needs to be defined explicitly. Were the Dynabeads continuously present, or were they removed and reintroduced at each cycle? This distinction is important for interpreting the stimulation regime.

We thank the reviewer for highlighting this point. To clarify the experimental procedure, we have revised the Materials and Methods section as follows:

"Cells were re-stimulated with Dynabeads every two days for 8 days to induce chronic stimulation, with previously added beads removed and replaced with fresh beads at each restimulation."

This clarification explicitly distinguishes the protocol from continuous bead exposure and more accurately describes the repeated stimulation regimen used throughout the study.

7. Presentation and typographical errors.

The manuscript still contains multiple typographical and presentation issues that should have been resolved at the revision stage.

In the merged main text and figure file:

Line 511: stimulation

Line 539: at Line 551: mCherry

Line 583: Leukocyte

Lines 656 and 671: promoters

Fig. 7H:

In the supplementary file:

We thank the reviewer for his attention to these typographical errors. We have now fixed them all. Regarding the Figure 7H: TEX, there is no issue.

Overall, while the revision improves some aspects of the manuscript, the central concerns remain: the platform claim is overstated, the experimental validation is too narrow, and the most physiologically relevant systems are not examined with sufficient rigor. For these reasons, I remain unconvinced that the manuscript reaches the level of novelty and completeness required for Nature Communications.

Reviewer #2 (Remarks to the Author):

The authors have addressed all of our comments.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4 (Remarks to the Author):

Thanks for the extensive revisions and the additional data. The revised version substantially enhances the paper.

I do find the paper adequate to publish.

Realizing other reviewers had some less favorable comments and overall appreciation, I

must note the study is original, interesting, and will have an impact. It is, in my opinion, not fair to dismiss such a good study because other related papers are published- this is true for >90% of good papers, and we must publish solid research that brings new data, even if related studies are present. Basic, fundamental science must have a place to publish and communicate findings that will progress our society.

I encourage the authors to get it published as it should be.

We sincerely thank the reviewer for the careful evaluation of our manuscript and for the encouraging comments. We greatly appreciate the recognition of the originality, potential impact, and broader significance of this work. We are particularly grateful for the reviewer's acknowledgment of the extensive revisions and additional experiments performed, as well as for recognizing the value of fundamental and tool-building research in advancing the field. We thank the reviewer for the positive assessment and support for publication.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have further revised the manuscript and performed additional experiments to address the concerns raised during the previous round of review. They have now satisfactorily addressed major comments 1 and 3, as well as all of my previous minor comments.

However, I remain only partially convinced by the authors' response to major comment 2 concerning novelty. Although the manuscript now provides a more comprehensive in vivo characterization of NR4A2-based synthetic promoters (SPs) than previous studies, these advances are still focused on an already established SP. Other SPs beyond the NR4A- and NFAT-based systems, which are likely to be of considerable interest to the community, were neither characterized to a comparable extent nor directly benchmarked against the NR4A2-based SP. What architectures presented here could be readily adapted for applications beyond T-cell engineering, the manuscript would provide substantially greater value to the field if the authors deposited a plasmid toolkit containing the principal SP constructs described in the study in a publicly accessible repository.

We thank the reviewer for the positive evaluation of the revised manuscript and are pleased that the additional experiments have satisfactorily addressed the major scientific concerns. We also appreciate the suggestion regarding broader dissemination of the synthetic promoter toolkit. To facilitate reproducibility and reuse, we have deposited all synthetic promoter sequences in GenBank (accession numbers provided in the revised manuscript), and detailed cloning procedures are described in the Methods. While we have not deposited the plasmids in a public repository at this stage, we appreciate the reviewer's suggestion and will consider depositing the complete plasmid collection in Addgene in the future to further facilitate community access.

Minor comments

1. Figure 7H: Exhausted T cells are conventionally abbreviated as TEX rather than TTEX. Alternatively, if the authors prefer the term "dysfunctional T cells," an abbreviation such as Tdysf would be appropriate. Regardless of the terminology chosen, the full term and its abbreviation should be introduced clearly and used consistently throughout the manuscript.

We thank the reviewer for this observation. We used the abbreviation "TTEX" to specifically indicate *terminally exhausted T cells*, referring to the subset co-expressing multiple exhaustion markers (LAG-3, TIGIT, CD39, PD-1, NR4A2 and TIM-3), following the recommendation of our immunology collaborator Prof. Luca Gattinoni. Our intention was to distinguish this population from the broader TEX compartment.

2. The description of Figure 7H in the main text is not fully supported by the data presented. Specifically, Figure 7H does not appear to show an analysis of the relationship between mCherry expression and exhaustion-marker expression. Furthermore, the gating strategy used to identify exhausted T cells within the tumour should be described in sufficient detail. The statement that

“multiple exhaustion markers were combined to determine...” is too vague and should be replaced with a precise description of the markers used and the gating strategy applied.

We thank the reviewer for pointing out this discrepancy. We have revised the text describing Figure 7H to accurately reflect the analysis presented. Specifically, the panel shows the Pearson correlation between the percentage of mCherry-positive cells and the frequency of terminally exhausted CD8⁺ T cells. We have also expanded the description of the gating strategy in the manuscript. Following gating on live CD8⁺ T cells, LAG-3⁺TIGIT⁺ cells were first identified, followed by selection of CD39⁺PD-1⁺ cells, and finally NR4A2⁺TIM-3⁺ cells within this population, which were defined as terminally exhausted (TEX) CD8⁺ T cells. The gating strategy is now described in detail in the Results section and Methods.

3. Details of the antibodies used to detect the endogenous transcription factors MAF, IRF4, BATF, GATA3, IKZF2, and EOMES in primary T cells are missing and should be included in the Methods or Supplementary Information.

We have now included the details of all antibodies used for intracellular detection of endogenous transcription factors in the Materials and Methods. Briefly, cells were permeabilized using the Transcription Factor Buffer Set (BD Pharmingen™) and stained individually with the following antibodies: anti-IRF4-AF488 (clone IRF4.3E4, Cat. No. 3832030, Sony), anti-BATF-PE (clone 9B5A13, Cat. No. 3874015, Sony), anti-MAF-PE-eFluor610 (clone sym0F1, Cat. No. 61-9855-42, Invitrogen), anti-GATA3-BV421 (clone W19195B, Cat. No. 2534560, Sony), anti-NR4A2-Coralite CL488 (Cat. No. CL488-66878, Proteintech), anti-EOMES-PE-Cy7 (clone WD1928, Cat. No. 25-4877-42, Invitrogen), and anti-IKZF2-PE-Cy5 (clone 22F6, Cat. No. 15-9883-42, Invitrogen).