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Original Research Article

Improving cooperativity of transcription activators by oligomerization domains in mammalian cells

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A B S T R A C T

Cooperative activation is critical for the applications of synthetic biology in mammalian cells. In this study, we have developed cooperative transcription factor by fusing oligomerization domain in mammalian cells. Firstly, we demonstrated that two oligomerized domains (CI434 and CI) successfully improved transcription factor cooperativity in bacterial cells but failed to increase cooperativity in mammalian cells, possibly because the additional mammalian activation domain disrupted their oligomerization capability. Therefore, we chose a different type of oligomerized domain ($CarH_C$), whose ability to oligomerize is not dependent on its C-terminal domains, to fuse with a transcription factor (RpaR) and activation domain (VTR3), forming a potential cooperative transcription activator RpaR-CarH-VTR3 for mammalian regulatory systems. Compared with RpaR-VTR3, the cooperativity of RpaR-CarH-VTR3 was significantly improved with higher Hill coefficient and a narrower input range in the inducible switch system in mammalian cells. Moreover, a mathematical model based on statistical mechanics model was developed and the simulation results supported the hypothesis that the tetramer of the CarH domain in mammalian cells was the reason for the cooperative capacity of RpaR-CarH-VTR3.

1. Introduction

The complex regulation of eukaryotic promoters relies on the cooperativity of multiple transcription factor complexes to initiate transcription [1,2]. In mammalian cells, most promoters expressed by RNAP II require numerous transcription factors to stimulate transcription cooperatively [3–5]. The cooperative regulation of transcription factors is key to mammalian cell development [6,7], signal processing [8,9], cell fate decisions [10], non-linear regulatory operations and sensitivity [11] of regulatory networks. Cooperative activation enables the construction of switches with ultrasensitive responses, which are widely used in mammalian cells for synthetic biology applications such as gene therapy, cell fate editing, and eukaryotic cell factories [12–15]. To date, most engineering strategies for the cooperativity of transcription activators in eukaryotic cells have relied on complex protein scaffolds [16] and tandem activation domains [17–19], which limit the composability and engineering behavior of the system.

The multimerization of transcription factors is a modular and efficient cooperative regulation mechanism of prokaryotic cells. Engineered transcription factors fused with multimerization domains showed enhanced cooperativity in prokaryotic cells such as *E. coli* [20]. However, fusing such prokaryotic multimerization domains with transcription activators to enhance activation cooperativity in mammalian cells remains largely unexplored. We wondered whether cooperative mammalian activation systems could be achieved through fusing transcription activators with multimerization domains of prokaryotic transcription factors. First, the multimerization domains of transcription factors were found to be modular and can be used to improve cooperativity of transcription factors [21]. What's more, the multimerization domains of prokaryotic transcription factors are usually small, which might significantly reduce the load of expression on cells and increase carrier capacity.

Here, we explored the activation and synergistic effects of fusion expression of three different multimerization domains with the transcription factor RpaR-VTR3 in mammalian cells. We achieved the engineered RpaR-VTR3 with potential for cooperativity and sensitivity

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by expressing CI434, CI, CarH and promoter architecture respectively, and further utilized the small molecule responsiveness of RpaR to construct a highly sensitive switch. We developed a theoretical explanation for multimerization domains on cooperativity and sensitivity of genetic switch using a statistical thermodynamic model.

2. Results

2.1. The CI and CI434 oligomeric domains did not increase the cooperativity of transcription factor RpaR-VTR3 in mammalian cells

Inspired by the modular multimerization domain in *E. coli*, we first selected the oligomerized domains of CI and CI434 protein and fused them to the middle of RpaR-VTR3, which was dimeric but lacks high

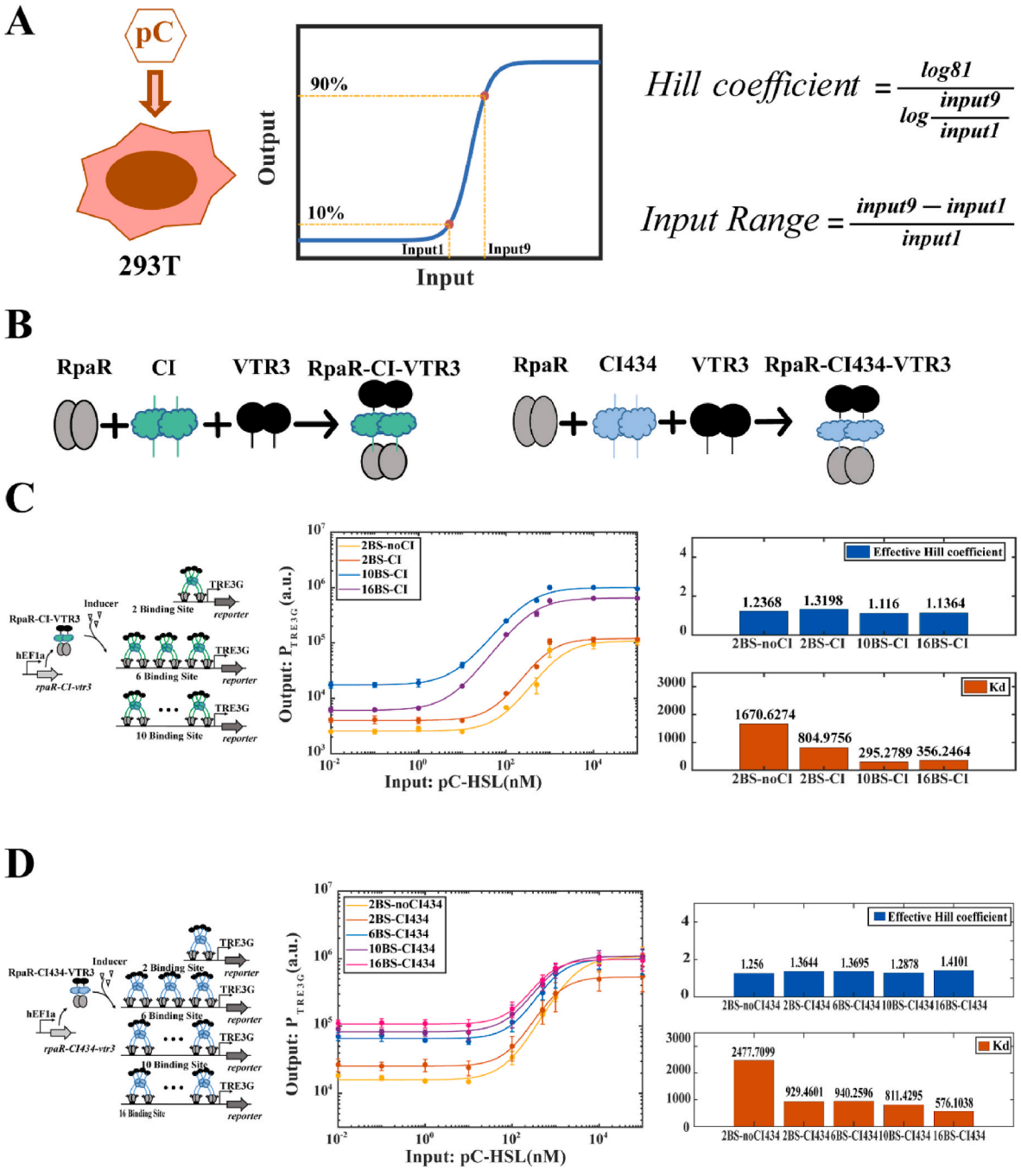

Fig. 1. The design and evaluation of the CI and CI434 oligomeric domains.
(A) Is the schematic representation of inducible switch. pC-HSL as inducer was added to HEK293T cell lines and triggered RpaR to bind to operator sequences upstream of TRE3G promoter sequence. Hill coefficient and input range were derived from measurements to evaluate cooperativity of engineered activators. **(B)** Is the schematic representation of chimeric transcription activators. Engineered transcriptional activators were constructed by fusing VTR3 domain and cooperative domain (CI/CI434) to the N-terminal of RpaR. **(C)** and **(D)** is the design and evaluation of the CI and CI434 oligomeric domains. RpaR-CI434-VTR3 hybrid transcriptional activator was expressed by hEF1α promoter and promoter architectures were tuned using varied number of operator sequences (BS). The CI and CI434 oligomeric domains did not increase the cooperativity of transcription factor RpaR-VTR3. The experimental points on the dose-response curves are the mean of at least three biological replicates.

oligomerization ability in mammalian cells (Fig. 1 B&C). Fused to the C-terminus of RpaR, CI or CI434 protein, as oligomerized domain, was inserted between RpaR DNA-binding domain and VTR3 activating domain [22] (Fig. 1B). Engineered transcription factors RpaR–CI–VTR3 (RpaR-VTR3 fused CI domain) and RpaR-CI434-VTR3(RpaR-VTR3 fused CI434 domain) were respectively overexpressed by the constitutive promoter Hef1α. Two operators were placed upstream of the regulated promoter TRE3G to facilitate the binding of each RpaR-CI434-VTR3 or RpaR–CI–VTR3 dimer to each CarO operator, and a reporter gene(sfgfp) was used to measure the regulated promoter activity. Meanwhile, we measured the promoter activity regulated by RpaR-VTR3 with the same concentration gradient inducer pC-HSL, compared to promoters regulated by RpaR–CI–VTR3 and RpaR-CI434-VTR3. Hill coefficients and input range were used to evaluate the two engineered transcription factors RpaR-CI434-VTR3 and RpaR–CI–VTR3 for cooperativity (Fig. 1A). We expected that the two engineered RpaR-CI434-VTR3 and RpaR–CI–VTR3 would cooperatively bind to the two CarO operators, and improve their activation capability and ultrasensitivity for the regulated promoter activity, with higher Hill coefficient and a narrower input range (Fig. 1B&C). However, after analyzing experimental measurement data, we found that the curves corresponding to RpaR–CI–VTR3 and RpaR-CI434-VTR3 did not display significant cooperativity compared to the control group. Both hill coefficient and input range of the curves fitted from experimental measurement data also indicated that the oligomerization domain of the CI and CI434 protein did not work in this case (Fig. 1 B&C). The results of the experimental data were similar to those from the previous ones, even though we kept increasing the number of upstream operators of the TRE3G promoter. The two engineered RpaR-CI434-VTR3 and RpaR–CI–VTR3 did not improve activation capability and ultrasensitivity for the regulated promoter activity.

After analyzing the structures of the oligomerized domain of the CI and CI434 protein, we found that the cooperative DNA binding of the CI and CI434 protein in prokaryotes is mediated by a group of residues in the C-terminal domain (CTD) [23] and hypothesized that the fused VTR3, which is a crucial activating domain in mammalian cells, destroyed the modular oligomerized domain of the CI and CI434 protein, resulting the two engineered RpaR-CI434-VTR3 and RpaR–CI–VTR3 did not work as we expected. Therefore, owing to the differences of transcription regulation between prokaryotic and eukaryotic systems, the modular oligomerization domain of CI and CI434 are not satisfied in mammalian cells, and we need to find a more modular oligomerized domain protein.

2.2. The CarH protein domain increased the cooperativity of transcription factor RpaR-VTR3

Based on analysis about the oligomerized domain of the CI and CI434 protein, the oligomerized domain of which the C-terminus is responsible for its self-assembly and cooperative DNA binding could not be fused to the transcription factors RpaR-VTR3 as an additional oligomerized domain in mammalian cells. We need to find other oligomerized domain proteins which can hold such multimerization facility after fused to VTR3. Photoresponsive protein hydrogels has been synthesized by covalently polymerizing the adenosylcobalamin (AdoB12)-dependent photoreceptor C-terminal adenosylcobalamin binding domain ($CarH_C$) proteins under mild physiological conditions [24,25]. The covalently cross-linked $CarH_C$ hydrogel which was polymerized through SpyTag-SpyCatcher chemistry can remain tetramerization. In fact, the CarH protein was a bacterial transcriptional regulator controlling bacterial carotenoid synthesis by as a tetramer binding operator DNA thus blocking transcription [26–29]. Moreover, CarH protein was used to construct the modular light-sensitive response system [30,31]. It seems that the CarH protein is what we are looking for to form multimerization transcription factors in mammalian cells.

To create the chimeric transcription factor RpaR-CarH-VTR3, which functions similarly to the previous regulatory systems RpaR–CI–VTR3 and RpaR-CI434-VTR3, we inserted CarH protein between RpaR and VTR3 (Fig. 2 top). The chimeric transcription factor RpaR-CarH-VTR3 were constitutively expressed by the constitutive promoter Hef1α and the reporter gene (sfgfp) was controlled by the promoter TRE3G with two RpaO operator in tandem with the pC-HSL inducers titrated was used to measure the regulated promoter activity. The dose-response curve would exhibit cooperative activation capabilities, ultrasensitivity, and a reduced input range if the CarH protein is responsible for the tetramer of the newly designed transcription factors RpaR-CarH-VTR3 under optimal conditions. The experimental results and our predictions were quite consistent (Fig. 2, bottom). With the oligomerized domain of CarH protein, the Hill coefficient of the transcription factor RpaR-CarH-VTR3 was increased by 89.7% (from 1.56 to 2.96) and the input range was decreased to 3.4 when compared with the RpaR-VTR3 without the oligomeric domain (Fig. 2, red line vs yellow line). The circuit was more sensitive to the inducer and could reach saturation of promoter expression within a smaller input range as the cooperativity of transcription activators increased. This preliminary evidence suggests that CarH acts as a cross-linked oligomerized domain to improve the cooperativity of transcription factor RpaR-VTR3. These results indicated that oligomerized domain of CarH protein was important for improving transcription factor cooperative capability in mammalian cells.

2.3. More than two operators did not improve the cooperativity of RpaR-CarH -VTR3

Although the experimental results indicated that the cooperativity of the chimeric engineered transcription factors RpaR-CarH-VTR3 was improved, we did not know whether the number of operators could result in a further improvement of cooperativity of RpaR-CarH-VTR3, besides tetramer formed on two operators by CarH. To verify the cooperative capability of RpaR-CarH-VTR3, a series of additional operators were placed upstream of the regulated promoter (Fig. 3 A&B). Output promoter activity activated by RpaR-CarH-VTR3 or RpaR-VTR3 was detected by the same reporter gene (sfgfp) in the presence of titrated pC-HSL inducers. To evaluate the contribution of the number of operators on their ultrasensitivity, Hill coefficients and input range were used again to characterize the slopes of the input−output response curves. Furthermore, the number of operators may simply increase the sensitivity of transcription factors RpaR-CarH-VTR3 as RpaR-CI434-VTR3 and RpaR–CI–VTR3. With the addition of a series of additional upstream operators, the experimental results of both RpaR-CarH-VTR3 and RpaR-VTR3 maintained their cooperative capability in tetramer transcription factors formed by CarH without significant further increase in Hill coefficient and no further decrease in input range (Fig. 3 A&B). This result indicated that the cooperativity of the chimeric engineered transcription factors RpaR-CarH-VTR3 in the previous experiment was from the tetramerization of CarH and was independent of the number of operators.

2.4. Biophysical model validates the cooperativity caused by RpaR-CarH-VTR3

To understand the improvement of cooperativity from a microscopic perspective, we developed a mathematical model based on previous statistical mechanics model [32–37] (SI Materials and Methods). And we made the following assumptions. Firstly, for transcription factors, inducer-binding reaction occurs considerably more quickly than transcription factors-inducer-operator reaction. As a result, the CarH-fused transcription factor does not affect the parameter of n, KD for inducer-binding reaction. Secondly, for transcription factor RpaR-VTR3, the parameter K2 is the equilibrium constant of the transcription factors-inducer binding to the single operator reaction and recruiting RNA polymerase to initiate. There is no interaction between the transcription factors, thus bounding to operator for transcription factors is

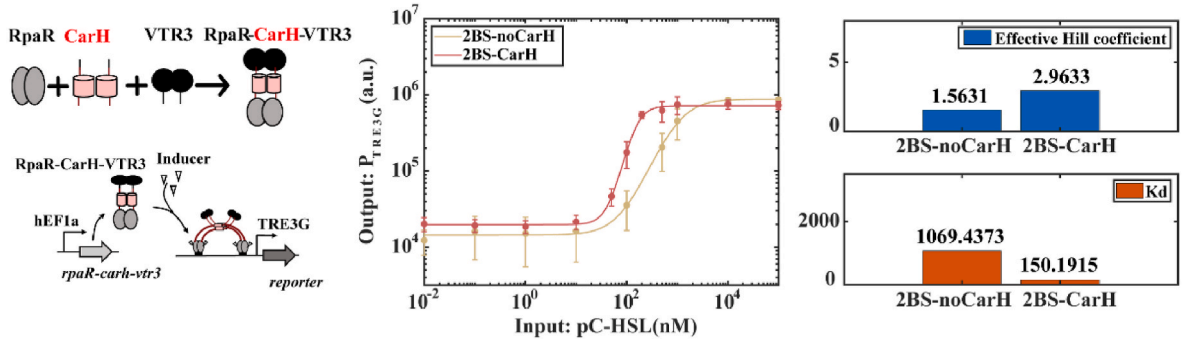

Fig. 2. The design and evaluation of the cooperative transcription factors RpaR-CarH-VTR3. RpaR-CarH-VTR3 was constructed by fusing VTR3 domain and cooperative CarH domain to the N-terminal of RpaR (left panel). Two operator sequences were placed upstream of TRE3G promoter sequence. The CarH oligomeric domains did increase the hill coefficient as well as decrease the input range (Kd) of transcription factor RpaR-VTR3 (middle and right panel). The experimental points on the dose-response curves are the mean of at least three biological replicates.

mutually independent. Thirdly, for transcription factors RpaR-CarH-VTR3, the parameter ω is used to characterize the cooperative capability, describing the cooperative process of the tetramer transcription factors RpaR-CarH-VTR3 bind to the second operators. The bigger is ω, the higher is the cooperativity of transcription factors RpaR-CarH-VTR3.

The total microscopic configurations and promoter activity probability of the two transcription factors (RpaR-CarH-VTR3 and RpaR -VTR3) are shown in the figure, respectively, where only two operators are present (Figure SI). Similarly, the transcription factors RpaR-CI434-VTR3 and RpaR–CI–VTR3 have similar total microstates and promoter activity. The promoter activity associated with the transcription factors RpaR-CarH-VTR3, RpaR-VTR3, RpaR-CI434-VTR3, and RpaR–CI–VTR3 was simulated using our model, respectively (Fig. 4). Every row in every column respectively corresponds to the normalized fitting curve, simulation curve and evaluation index for different transcription factors RpaR-VTR3, RpaR-CarH-VTR3, RpaR-CI434-VTR3 and RpaR–CI–VTR3. Hill coefficient, input range and dissociation constant were calculated for all the curves simulated by the model. It was found that only the curves of RpaR-CarH-VTR3 improved the cooperativity while the others remained intact when fusing with the modular oligomerization domain in *Escherichia coli* or none. As a result, the findings of the simulation demonstrate that the cooperativity is caused by the CarH protein domain rather than the number of operators (Fig. 4).

3. Discussion and conclusion

In this study, we addressed the possibility of developing cooperative transcription factor by fusing oligomerization domain in mammalian cells. Firstly, CI434 and CI, the modular multimerization domain in prokaryotic cells, were fused with transcription factors RpaR -VTR3 and titrated with the pC-HSL inducers. The expression of the reporter gene was measured by flow cytometry. There was no significant cooperativity improvement in the dose-response curves of transcription factors RpaR-CI434-VTR3 and RpaR–CI–VTR3 compared to RpaR -VTR3. By analyzing the protein structure, we hypothesized that the fusion with VTR3 might disrupt the C terminus of the oligomerization domain CI434 and CI, preventing it from maintaining its ability to self-assemble and cooperate with other DNA-binding proteins. Subsequently, based on the cross-linking protein domain CarH, we have developed the brand-new engineered transcription factors RpaR-CarH-VTR3 to regulate cooperativity of promoter activity, which were significantly increased with the improvement of the Hill coefficient and the reduction of the input range. In order to further verify the mechanism of the cooperativity of engineered transcription factors RpaR-CarH-VTR3, we have developed a biophysical model based on previous statistical mechanic model. Simulation studies indicate that the cooperativity is mediated by the CarH protein domain. This further confirmed our design developing cooperative transcription factors by fusing oligomerization domain CarH protein in mammalian cells has been realized.

It is challenging for synthetic biology to actualize the cooperativity of transcription factors with straightforward gene regulation due to the intricacy of transcriptional processes in eukaryotic cells. The existing cooperativity designs of eukaryotic cooperating transcriptional activators which include protein scaffolds, tandem multiple activation domains, the increase in the number of operators, could improve cooperative effect of transcriptional activator with using a large number of proteins at the same time, but introduced extra host burden of genetic systems, which would compromise circuit function. It is clear that these methods are not conducive to rationally design and construct complex systems in mammalian cells. We realize the improvement of cooperativity of transcription factors in mammalian cells with a small and exquisite cross-linking protein domain CarH. Clearly, our design reduces the burden on the host cell, is more likely to be modular in mammalian cells, and is better suited for the future design of regulatory networks for complex target functions in mammalian cells.

In our work, the chimeric engineered transcription factors RpaR-CarH-VTR3 were constructed using native and intact RpaR and CarH coding sequence, and flexible linkers were used to bridge the CarH protein domains and DNA binding/activation domain. The tetramerization of the chimeric transcription factor RpaR-CarH-VTR3 was caused by the covalently cross-linked CarH protein, and it did not affect the DNA binding/activating domain and function of the RpaR and VTR3, the function of CarH was not affected by the RpaR and VTR3, and vice versa. Therefore, the oligomerized domain of CarH protein did not affect the sensing and allosteric capability of the chimeric transcription factor. Therefore, the DNA binding domain/activating domain fused with the CarH protein could be capable of being replaced with other regulatory element to form multimerization transcription factors and increased the cooperativity of transcription factor. Thus, the construction strategy of improving the cooperativity of RpaR-VTR3 by fusing CarH could be extensive and modular.

In addition, we developed a biophysical model based on previous statistical mechanics model to understand the improvement of cooperativity from a microscopic perspective, and to validate that the CarH protein domain is what causes the cooperativity. This further confirmed our design developing cooperative transcription factors by fused an oligomerization domain CarH protein in mammalian cells has been realized. Our model was created by combining basic transcription factors and inducers with promoter activity characterized by the partition function. Additionally, the model and experimental findings were quite consistent. A statistical thermodynamic model has been used to understand the increase in cooperativity from a microscopic perspective. The chimeric-designed transcription factors RpaR-CarH-VTR3, however, are the only ones we have created thus far. Indeed, to achieve quantitative predictability of transcriptional regulation in mammalian cells,

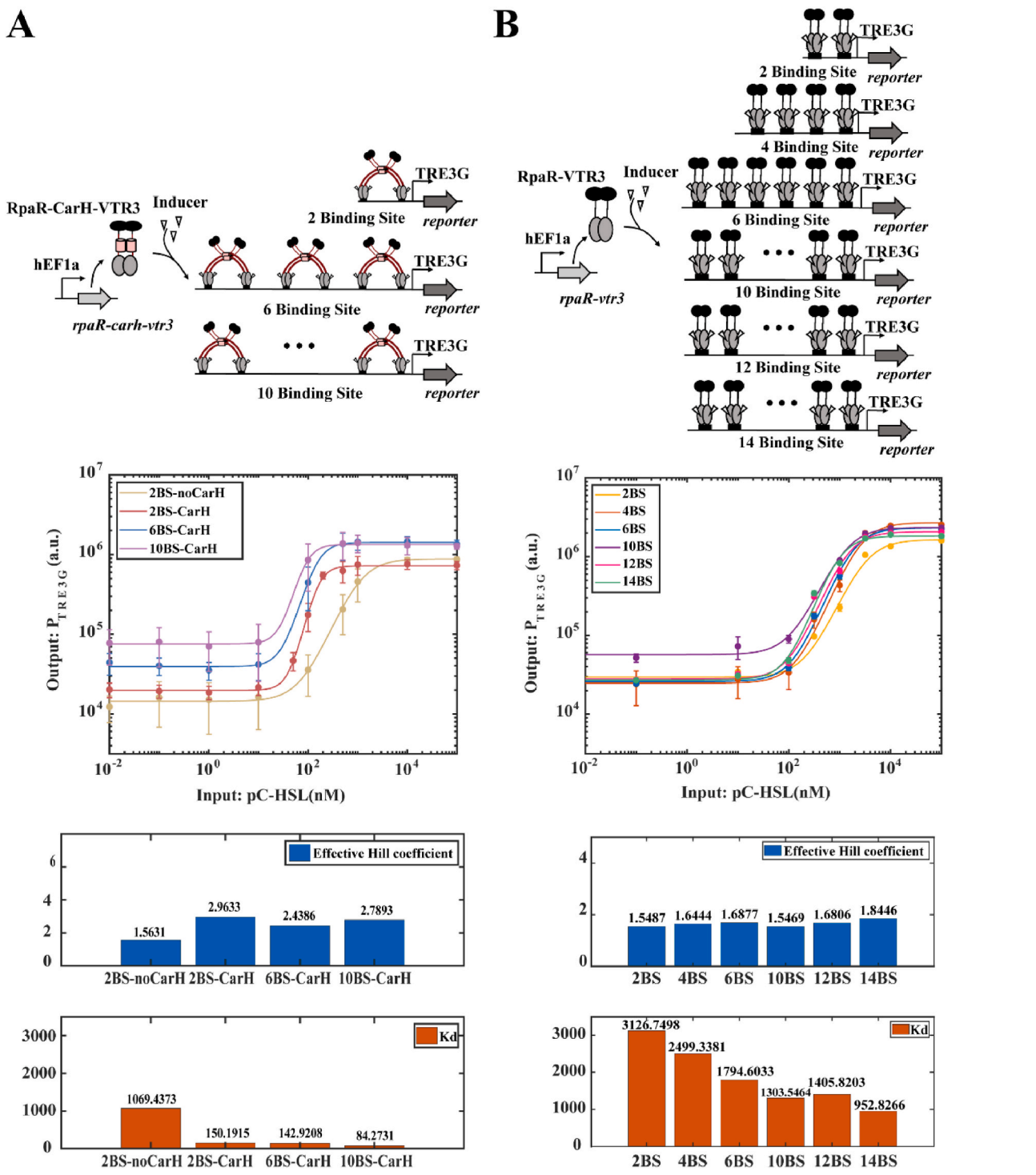

Fig. 3. The number of operators did not improve the cooperativity of transcription factors RpaR-CarH-VTR3. The experimental fitting results of both RpaR-CarH-VTR3 (a) and RpaR-VTR3 (b). RpaR-CarH-VTR3 might maintain their cooperative capability in tetramer transcription factors formed by CarH as Hill coefficient was not significantly further improved and the input range was not further decreased with the increase of operator sequence numbers. While the number of operators did not affect the cooperative capability of transcription factors RpaR-CI434-VTR3 and RpaR–CI–VTR3 and led to a slight decrease of input range. The experimental points on the dose-response curves are the mean of at least three biological replicates.

additional regulatory modules need to be exploited.

4. Materials and Methods

Cell culture. Human Embryonic Kidney 293T cells 286 (HEK293T, ATCC) were cultured in High Glucose Dulbecco's modified Eagle's media (DMEM-high glucose, Hyclone), and supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1% penicillin-streptomycin (Hyclone). All cell cultures were kept under 5% CO2 in 37 °C temperature.

Plasmid construction. Plasmids used in this study were derived from pRT vectors which were constructed and preserved in our laboratory[33]. pRT vectors were modified from plasmid PB531A-1 from the PiggyBac system. DNA sequences of plasmids used can be found in

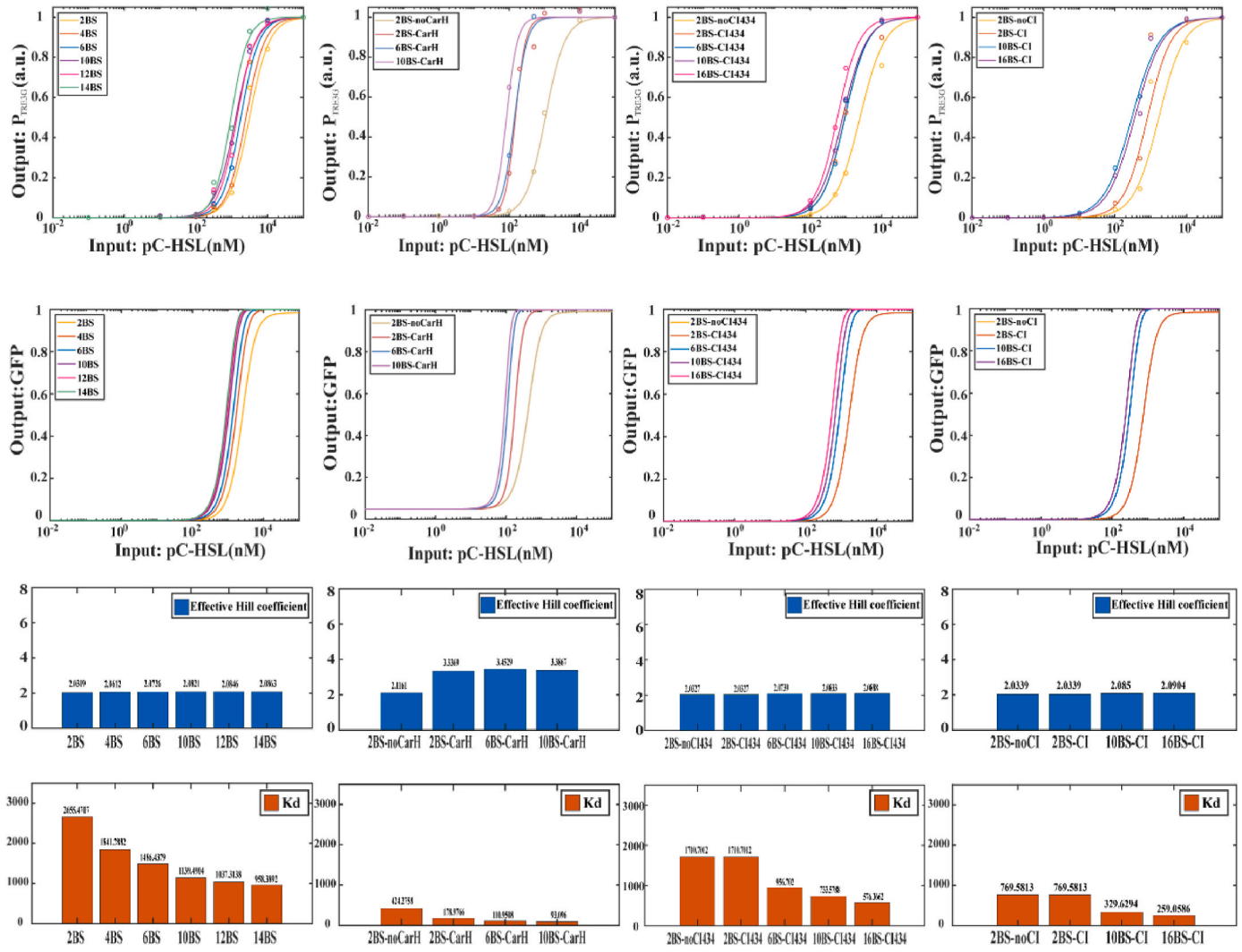

Fig. 4. The simulation results confirmed the improvement of the cooperativity caused by CarH protein domain. Every row in every column respectively corresponds to the normalized fitting curve, simulation curve and evaluation index for different transcription factors RpaR-VTR3, RpaR-CarH-VTR3, RpaR-CI434-VTR3 and RpaR–CI–VTR3. Simulation results implied that the cooperativity was caused by the CarH protein domain rather than the number of operators.

Supplementary Table. The coding sequences of fused proteins CI-RpaR/CI434-RpaR/CarH-RpaR were synthesized via gene synthesis (Shanghai Generay Biotech Co., Ltd.) and optimized for expression in mammalian cells. Inducible promoters activated by RpaR were created by placing the RpaR operator (RpaO) adjacent with TRE3G promoter sequence. Other basic parts (hEF1α promoter, 5′UTRs, 3′UTRs, and terminators) were created via standard cloning techniques. The final plasmids were constructed using a Golden Gate strategy. Plasmids were transformed into Top10 *E. coil* competent cells and these cells were plated on LB agar and propagated in LB media. All plasmids were extracted from cells with QIAprep Spin Miniprep.

Cell transfections and fluorescence measurement. All plasmids were transfected into cells by Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. 50 μg of each plasmid was transfected into CHO cells in 15 cm dishes. Cells were harvested after 48 h of transfection. 100 μL PBS was added to wash the cells, and 40 μL Trypsin-EDTA (GIBCO) was added. Then cells were suspended and transferred to 96 well plates. The plated were spun down at 200×*g*, 5 min, and the media was aspirated. Following this, Cells were resuspended in 200 μL 4% paraformaldehyde (PFA, Boster Biological Technology). 100,000 cells were analyzed for each sample on a Beckman Coulter CytoFlex S flow cytometry or BD Fortessa SORP equipped with proper lasers and filters, and data were analyzed with Matlab R2018a.

4.1. Biophysical Modeling and activation probability

The relative promoter activity probability of the four transcription factors with two operators was given as

$$P_i = \frac{K_1 + 2K_2 f_{TL} + \omega K_2^2 f_{TL}^2}{1 + K_1 + 2K_2 f_{TL} + \omega K_2^2 f_{TL}^2}$$

Where i could be RpaR-VTR3, RpaR-CarH-VTR3, RpaR-CI434-VTR3 or RpaR–CI–VTR3, f_{TL} was the fraction of the transcription factor bound to ligands, the parameter K_2 was the equilibrium constant of the transcription factors-inducer binding to the single operator reaction and recruiting RNA polymerase to initiate, and was fixed for four transcription factors. The parameter ω is used to characterize the cooperative capability, which was equal to 1 for RpaR-VTR3, RpaR-CI434-VTR3 and RpaR–CI–VTR3, and to 100 for RpaR-CarH-VTR. All parameters fitting and model simulation were executed by MATLAB R2018a, and parameters were listed in the supplementary tables.

CRediT authorship contribution statement

Xinmao Chen: Investigation, Methodology, Writing – original draft. **Ziming Liu:** Investigation, Methodology. **Chunbo Lou:**

Conceptualization, Supervision, Writing – review & editing, Funding acquisition. **Ying Guan:** Writing – original draft, Writing – review & editing, Investigation. **Qi Ouyang:** Funding acquisition, Supervision. **Yanhui Xiang:** Investigation, Methodology, Supervision.

Declaration of competing interest

We declare we have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.12.003.

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