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TALE activators regulate gene expression in a position- and strand-dependent manner in mammalian cells

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ABSTRACT

Transcription activator-like effectors (TALEs) are a class of transcription factors that are readily programmable to regulate gene expression. Despite their growing popularity, little is known about binding site parameters that influence TALE-mediated gene activation in mammalian cells. We demonstrate that TALE activators modulate gene expression in mammalian cells in a position- and strand-dependent manner. To study the effects of binding site location, we engineered TALEs customized to recognize specific DNA sequences located in either the promoter or the transcribed region of reporter genes. We found that TALE activators robustly activated reporter genes when their binding sites were located within the promoter region. In contrast, TALE activators inhibited the expression of reporter genes when their binding sites were located on the sense strand of the transcribed region. Notably, this repression was independent of the effector domain utilized, suggesting a simple blockage mechanism. We conclude that TALE activators in mammalian cells regulate genes in a position- and strand-dependent manner that is substantially different from gene activation by native TALEs in plants. These findings have implications for optimizing the design of custom TALEs for genetic manipulation in mammalian cells.

1. Introduction

Precise control of gene expression has broad applications for research, gene therapy, biotechnology, and synthetic biology [1–4]. Transcription activator-like effectors (TALEs) provide new molecular scaffolds as genome-editing tools featuring high programmability and specificity [5–7]. Originally discovered in the plant pathogenic bacteria *Xanthomonas sp.*, TALEs are composed of a customizable DNA-binding domain [8,9] that can be fused to an effector domain to carry out desired functions. For example, fusion to an endonuclease allows gene modification [10,11], whereas fusion to a known activator or repressor domain enables gene regulation [12,13]. TALEs have been used to modify endogenous gene expression in a number of systems, such as yeast [14], plants [15,16], and various mammalian cells including stem cells [1,2,17–19]. Despite its broad use, TALE-directed gene activation displayed varying levels of success in mammalian cells [1]. 22 of 26 published TALE activators failed to activate gene expression by at least fivefold [18]. Although combinations of TALEs can synergistically enhance gene activation, insight into the parameters governing TALE activity would clarify current inconsistencies and aid in the optimization of TALE design for more robust and fine-tuned genetic manipulation.

Previous studies on natural TALEs suggest that both binding site and strand are key parameters influencing TALE-mediated gene activation in plant cells. Native TALEs typically bind a target box in the promoter region of targeted genes, directing the transcription start site (TSS) 44–61 bp downstream of the DNA-binding sequence [20]. Positioning the TALE target box to different sites shifts the TSS accordingly, implying that TALEs activate transcription by directing the assembly of the transcriptional initiation complex [20]. Furthermore, natural TALEs appear to achieve *cis*-regulation only when bound to the sense strand, as a TALE box with a reverse-complement sequence did not induce the activity of the *Xanthomonas* AvrBs3 TALE [20].

However, studies on chimeric TALEs in mammalian cells point towards a different mode of gene activation. While no direct relationship between TALE activity and distance to the TSS has been established in mammalian cells, TALEs targeted to different regions

Abbreviations: C-ter, carboxy terminus; CMV, cytomegalovirus promoter; DBD, DNA binding domain; ED, effector domain; FBS, fetal bovine serum; GFP, green fluorescent protein; mCMV, minimal CMV promoter; NLS, nuclear localization signal; N-ter, amino terminus; TALE, transcription activator-like effector; TA, transcription activator; TBS, TALE binding site; TR, transcription repressor; TSS, transcription start site; T2A, self-cleaving 2A peptide sequence; UBC, ubiquitin C promoter; UTR, untranslated region.

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of the promoter exhibited significant variation in gene activation [1,18,19]. TALE activity in the mammalian system seems to require interactions at the promoter, since deletions in the promoter greatly reduced TALE-mediated expression of the oct4 gene in murine cells [19]. Epigenetic effects may be partially responsible for the inconsistencies in endogenous gene activation [12,19]. It remains largely unclear how TALEs interact with the targeted gene in the mammalian system, especially when the binding site is not situated within the promoter. Because of the potential layers of complexity, the determinants of TALE regulation in mammalian systems have yet to be systemically evaluated. With the rapidly expanding use of chimeric TALEs in mammalian cells, it is important to establish key parameters to guide TALE design.

In this study, we employed our recently developed EZ-TAL assembly and dual reporter system [21,22] to study the effect of target location and binding-strand selection on TALE activity in human embryonic kidney (HEK293) cells. By using a plasmid reporter system, we were able to study the role of individual parameters, thus minimizing epigenetic influences that could confound the results. Our results suggest that TALEs regulate genes in a position- and strand-dependent manner in the mammalian system.

2. Materials and methods

2.1. Design and construction of TALEs and reporters

All TALE constructs for use in mammalian cells were derived from the native hax3TALE of *Xanthomonas camperstris pv armoraciae*. Minimal C- (240 amino-acid) and N-terminal (183 amino-acid) regions flanking the central DNA binding domain were retained to preserve TALE activity, as previously reported [21]. For the mammalian TALE transcription activator (TALE-TA), a mammalian nuclear localization signal (NLS), followed by the synthetic transcription activation domain of VP16 [23,24], was placed at the C-terminus (Fig. 1A, upper panel). Similarly, the TALE transcription repressor (TALE-TR) was constructed by placing a mammalian NLS at the C-terminus, followed by synthetic transcription-dominant repressors derived from TIEG2 motifs [25] (Fig. 1A, middle panel). A TALE lacking a mammalian effector domain was also constructed (Fig. 1A, lower panel). To assess background GFP and luciferase activity, a mock control plasmid lacking TALE was co-transfected with the dual reporter plasmid.

Customized TALE repeat domains were synthesized by hierarchical ligation of individual monomers using the EZ-TAL Assembly Kit (SBI, Mountain View, California) according to the user manual. Dual-reporter plasmids harboring TALE-binding sites were generated by inserting sequences containing the TALE binding sites (TBS) at locations specified in the text and illustrated in Figs. 1–4.

2.2. Cell culture and transfection

Human embryonic kidney cells (HEK293) were maintained in high glucose Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FBS, 2 mM GlutaMax (Life Technologies, Carlsbad, CA), 100 U/ml penicillin, and 100 U/ml streptomycin. All transfections were performed in 6-well plates seeded with 2×10^5 cells the day before transfection. At 30–50% confluencey, cells were transfected with the reporter plasmid and the respective TALE construct, using Purefection transfection reagent according to the user manual (SBI, Mountain View, California). All transfection experiments used 2 μg of TALE expression plasmid DNA with 1 μg of reporter plasmids.

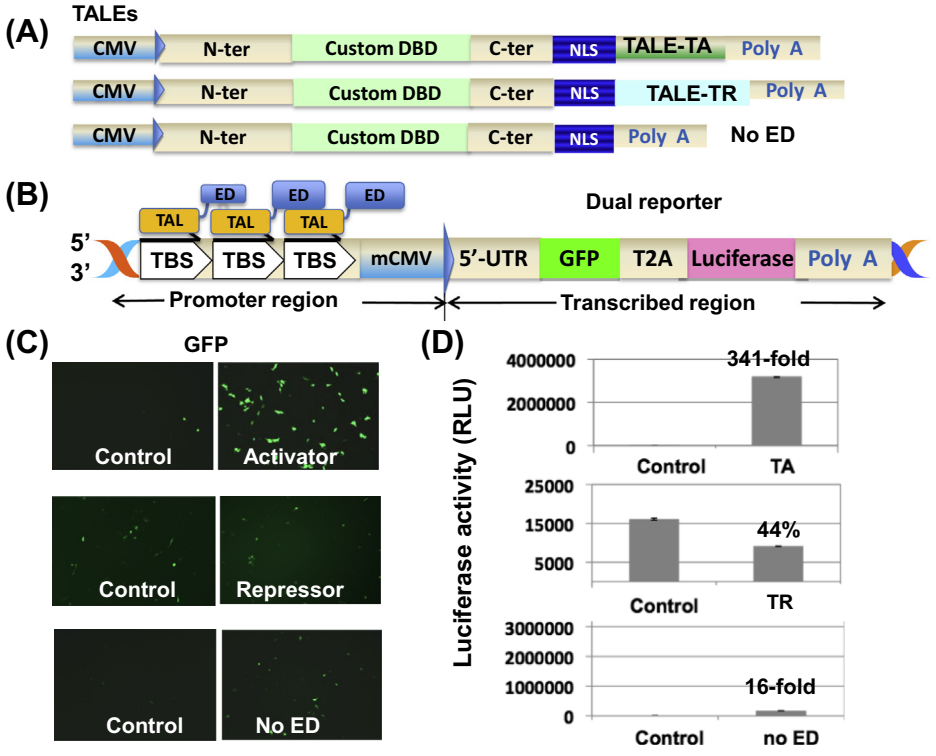

Fig. 1. TALEs and their effects on gene regulation. TALEs were co-transfected with dual reporters into HEK293 cells, and TALE activity was inferred from GFP and luciferase activity. (A) TALEs were designed to contain an activator domain (TALE-TA, upper panel), a repressor domain (TALE-TR, middle panel), or no effector domain (no ED, lower panel). Each TALE is driven by a CMV promoter, and contains a custom DNA binding domain (DBD), flanked by N-terminal and C-terminal regions, and a nuclear localization sequence (NLS). (B) Three copies of the TBS were inserted in sense-orientation into the promoter region of the dual reporter, enabling TALEs to bind and regulate the expression of GFP and luciferase. According to (C) GFP and (D) luciferase data, TALEs exerted the expected effects on gene regulation. Comparisons were done with a mock control, transfected with the same amount of plasmid DNA, but with an empty vector instead of the TALE.

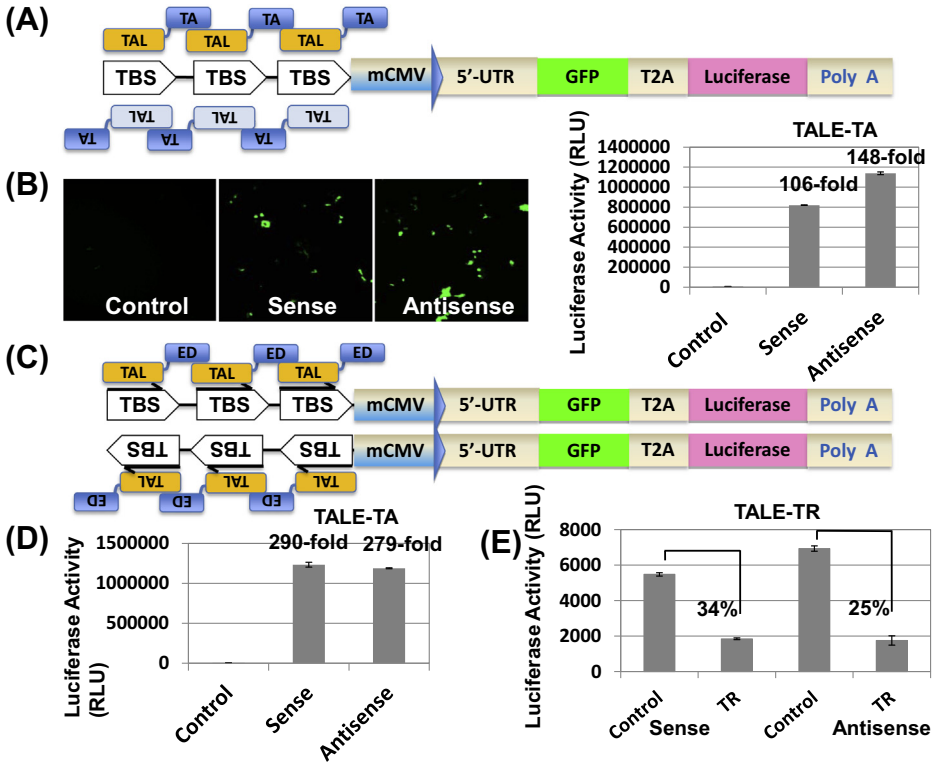

Fig. 2. Activation and inhibition by TALE effectors targeting the promoter region is strand-independent. (A) TALE-TAs were designed to bind to the sense or antisense strand of three TBS cloned into the promoter region of the dual reporter. (B) TALE-TAs activated the expression of GFP (left panel) and luciferase (right panel). (C) The three TBS in the promoter region of the dual reporter were cloned into the opposite orientation. (D) Independent of the TBS orientation within the promoter region, TALE-TA resulted in increased and (E) TALE-TR in decreased luciferase activity.

2.3. Microscopy and reporter gene assay

All microscopy was performed on live cells in 6-wells after 18 h after transfection. Cells were imaged using a LEICA DMI3000B microscope. Data collection and processing were performed with LAS 3.8 software. For the reporter luciferase assay, cells were lysed on plate and harvested 18 h after transfection. The lysate was cleared by centrifugation, and luciferase activity was measured with a BioTeck Synergy HT plate reader using a luciferase assay kit according to the manufacturer's instructions (Promega, Madison, WI).

2.4. Data collection and presentation

For live cell monitoring, cultured cells were monitored at indicated time points under a fluorescent microscope. GFP live cell images were taken using the same exposure condition and magnification within the group of comparison. For the luciferase reporter assay, all data are presented as mean ± S.D. ($n = 3$), unless stated otherwise.

3. Results and discussion

3.1. Design of mammalian TALEs and their corresponding reporters

The ability to specifically control gene expression has far-reaching applications in basic and applied biomedical research. Recent studies have revealed the potential for chimeric TALEs to activate endogenous genes in the mammalian system [1,2,12,17–19]. For example, a proof-of-concept study used custom TALEs to increase the transcription of the human frataxin gene, of which low expression causes nervous system damage in Friedreich's ataxia [17]. However, activation by different TALE activators differed greatly within mammalian systems, with the majority of TALEs displaying only modest gene activation [12,17,18,23,26]. Thus, the broad use of TALEs is currently limited by a tenuous understanding of parameters mediating TALE activity. The critical role of binding site location and binding strand in plant TALEs [20,27,28] suggests that insight into these factors may help resolve current inconsistencies in mammalian TALE activity.

We generated custom TALE-TAs to investigate the influence of TALEs on gene activation in a systematic manner. To reduce epigenetic influence, we used a previously established reporter system as a read-out [21]. We directed the TALE binding to either the promoter or the transcribed region of the dual reporter. In addition, we engineered TALE versions binding either the sense or antisense strand of the dual reporter in order to study the effects of binding strand choice on gene activation. All comparisons were made to a mock control transfected with the same amount of plasmid DNA, replacing the TALE with an empty vector.

3.2. TALEs activate reporter plasmids when binding the sense or antisense strand in the promoter region

To study the effects of TALEs on gene expression, we performed a co-transfection with TALEs and dual reporter plasmids in human embryonic kidney (HEK293) cells. As shown in Fig. 1A, we first generated three TALEs with different effector domains: a VP16 activator domain (TALE-TA), a repressor domain containing the TIEG2 motifs (TALE-TR), and a TALE lacking a mammalian effector domain. Each TALE contains a DNA-binding domain designed to recognize a 14 bp target within the Sox2 promoter as the TALE binding site (TBS) [21]. Three tandem repeats of this TBS were cloned 5′ adjacent to the mCMV promoter region of a dual reporter (Fig. 1B). As expected, co-transfection of the TALE-TA with the dual reporter drove

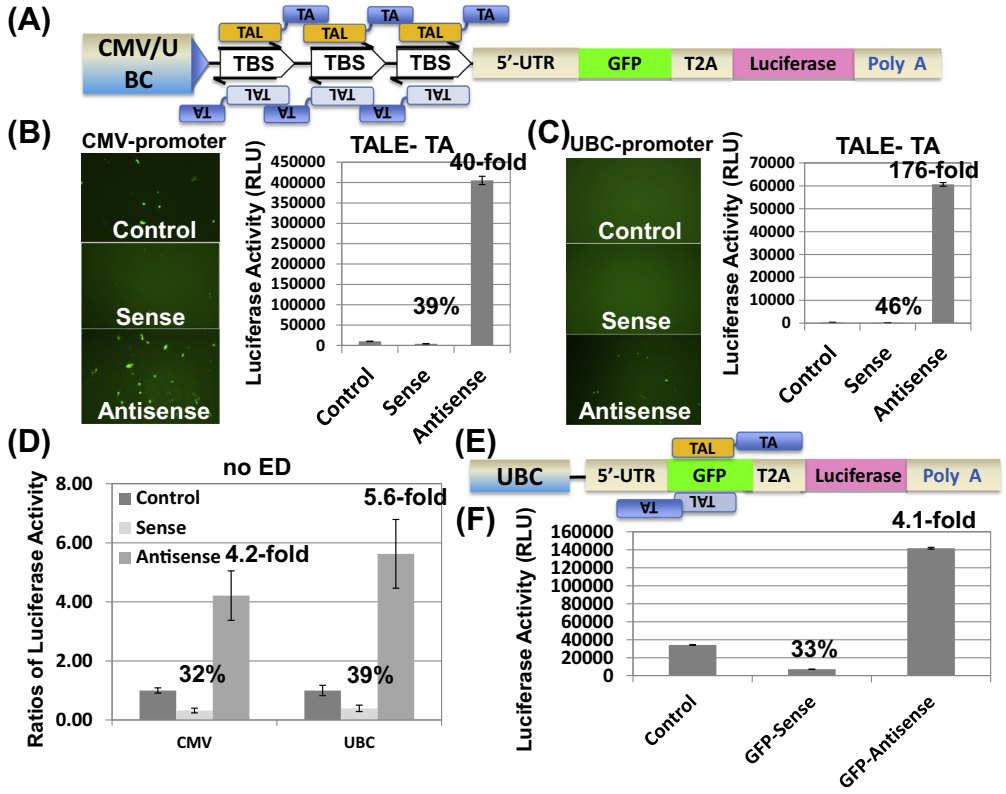

Fig. 3. TALE-TAs binding the transcribed region inhibit gene expression when binding the sense strand. (A) TALE-TAs were designed to bind either the sense or antisense strand of the TBS located in the 5′-UTR of the dual reporter gene construct, downstream of a viral CMV (upper panel) or non-viral UBC promoter (lower panel). Under control of the CMV promoter (B) or the UBC promoter (C), TALE-TAs exhibited significant repression when binding to the sense strand of the 5′-UTR. TALE-TAs binding the antisense strand retained their gene activation activity. (D) Similar inhibition was found with TALEs lacking an effector domain when binding to the sense strand, while slight activation was observed when binding the antisense strand. (E) TALE-TAs were designed to bind directly to the GFP-coding sequence of the dual reporter in sense or antisense orientation. (F) TALE-TA binding to the sense strand within the GFP coding region led to a decrease of luciferase activity, while binding the antisense strand increased luciferase activity.

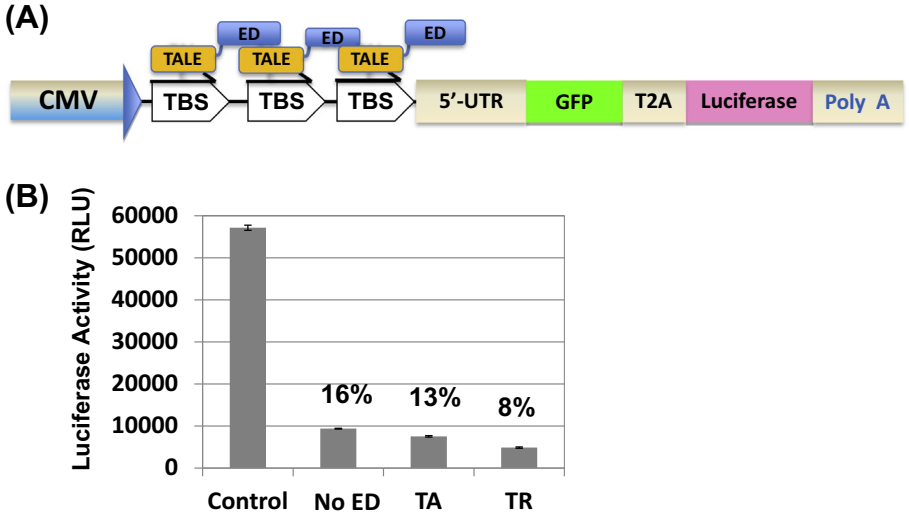

Fig. 4. The inhibitory effect of TALEs binding the transcribed region is independent of the effector domain. (A) TALEs with an activator, repressor, or no effector domain were designed to bind a TBS located in the 5′-UTR of the dual reporter. (B) Luciferase activity was repressed in all three TALE constructs, compared to the control.

a robust increase of GFP-positive cells (>85%), compared to a few weakly GFP-positive cells for the mock control (Fig. 1C, upper panel). In contrast, cells co-transfected with the TALE-TR and dual reporter displayed reduced GFP-activity, compared to the mock control (Fig. 1C, middle panel). Quantification via luciferase assays confirmed these trends, with a 341-fold increase in luciferase activity for TALE-TA and a 44% decrease for TALE-TR, compared with respective controls (Fig. 1D, upper and middle panels). The data show that our TALE-TA acts as a transcriptional activator and TALE-TR as a transcriptional repressor, as expected from prior knowledge of the effector domains [10,23,25]. Consistent with a previous study showing residual activity in TALEs with a deleted

activation domain [23], the TALE lacking an effector domain exhibited detectable GFP and a 16-fold increase in luciferase activity, compared to the control (Fig. 1C and D, lower panels). Because the only difference among the TALEs tested is the effector domain, these data show that chimeric TALEs containing different functional domains elicit corresponding effects on gene regulation when their binding sites target the upper sense strand in the promoter region. These results are in agreement with other studies that used a promoter sequence as the TALE target site [12,19,29].

After system validation, we investigated the strand dependence of TALE activity. We generated a TALE-TA to bind the reverse-complement sequence of the TBS, hence targeting the antisense strand at the same site of the reporter (Fig. 2A). The sense and antisense-strand binding TALE-TAs exhibited comparable activity, with 106-fold luciferase activation by the TALE-TA binding the sense strand and 148-fold activation by the TALE-TA binding the antisense strand (Fig. 2B).

Next we assessed the possibility that the different binding sequences of the sense and antisense TALE-TAs could influence TALE-TA activity. To eliminate this difference, we cloned an identical binding sequence into the same location, but in opposite orientation. Consequently, we were able to study the same TALE-TAs, binding its TBS either on the plus or on the minus strand, depending on the reporter construct used (Fig. 2C). In this more stringent comparison, co-transfection of sense and antisense-strand binding TALE-TAs with corresponding reporter plasmids resulted in remarkably similar activation of reporter genes (Fig. 2D). To test for possible strand-dependence of TALE-TR repressor activity when binding the promoter region, we repeated the above experiment using TALE-TR instead of TALE-TA. As can be seen in Fig. 2E, repression by TALE-TR was not affected by the binding strand.

Our results are consistent with a previous study that found no difference in TALE activity for mammalian TALE-TAs targeted to either strand of the VEGFA gene [18]. The observation that TALE-TAs strongly upregulated gene transcription when bound to either the sense or antisense strand of the TBS in the promoter region indicates that TALEs in the mammalian system act in a manner that is substantially different from the directional, strand-dependent activation observed in plants [20,28]. This finding demonstrates that TALE-TAs require design guidelines for mammalian systems that are different from those established for plants, to ensure proper specificity and activity in mammalian cells.

3.3. TALEs exhibit different activity when binding the sense or antisense strand in the transcribed region

Next, we determined if other regions besides the promoter could serve as candidate sites for TALE-mediated gene activation. Accordingly, we generated TALE-TAs binding the transcribed region and assayed their activity. To allow the detection of potential stimulatory or inhibitory effects, we replaced the minimal cytomegalovirus (CMV) with a strong CMV promoter to drive robust, constitutive gene expression. With the TBS placed just downstream of the promoter (Fig. 3A, upper panel), we found a significant inhibition of the reporter genes, as co-transfection resulted in a 39% decrease in luciferase activity for the TALE-TA binding the sense strand. In contrast, the TALE-TA binding the antisense strand upregulated luciferase by 40-fold (Fig. 3B, right panel). Consistent with the luciferase data, GFP activity in the antisense-binding TALE-TA showed increased intensity compared to the sense-binding TALE-TA (Fig. 3B, left panels).

To rule out the possibility that the TALE-mediated inhibition is due to promoter-specific interactions, we next generated a reporter driven by the ubiquitin C (UBC) promoter, which confers a moderate activity as compared to the CMV promoter. We found that co-transfection with the TALE-TA maintained a similar pattern of reporter gene expression, with a 46% decrease for the TALE-TA binding the sense strand and a 176-fold increase for the TALE-TA binding the antisense strand as quantified by luciferase activity (Fig. 3C, right panel), and confirmed by corresponding GFP data (Fig. 3C, left panels). Consistent with the residual activity of TALEs with a deleted effector domain [23], our TALE lacking an effector domain exhibited a slight increase in luciferase activity when binding the antisense strand of the promoter region (Fig. 3D), while binding the sense strand resulted in inhibition of lucifearase activity (Fig. 3D).

Having found that TALEs can repress gene expression when binding the sense strand within the 5′-UTR, we asked whether the inhibitory effect was restricted to the 5′-UTR of the transcribed region. To test this, we designed a TALE-TA binding the sense strand within the GFP coding region and co-transfected this TALE-TA with our dual reporter construct (Fig. 3E). We found a 33% decrease in luciferase activity compared to the control when targeting the sense strand of the GFP coding region, while binding the antisense strand in this region resulted in slight (4-fold) activation (Fig. 3F). These findings indicate that TALE-TAs binding the sense strand of the transcribed region can exert an inhibitory effect that is independent of the exact location.

3.4. The inhibitory action of TALEs binding the transcribed region is independent of the effector domain

To gain insight into the mechanism of repression, we tested TALEs with different effector domains designed to bind the 5′-UTR of the dual reporter (Fig. 4A). Upon co-transfecting TALEs containing an activator, a repressor, or no effector domain, we detected decreased luciferase activity of 13%, 8%, and 16%, respectively, for all constructs, compared to the control (Fig. 4B). These data show that the inhibitory activity of TALEs binding the 5′-UTR is independent of the effector domain. We speculate that this inhibitory effect may be due to tight binding of TALEs to their target DNA, physically preventing transcription. With the DNA binding domain of TALEs residing in the major groove of the targeted DNA [30], it may be quite difficult to dissociate the bound TALE, thus hindering transcriptional activity. Our results are consistent with the blockage activity of TALEs observed in other organisms. For example, it has been proposed that the binding of TALEs to the *lac* operator in bacteria blocks RNA polymerase from recognizing the promoter and subsequently initiating transcription [31]. Gene repression by TALEs lacking an activation domain has also been found in yeast [14]. Considering that blockage mechanisms by TALEs have previously been observed in bacteria and yeast, we conclude that TALEs can block transcription in mammalian cells when binding the sense strand of the transcribed region.

TALEs are relatively novel molecular tools, and they prove complex in their mechanism and function. As expected, TALEs binding the promoter regulate gene expression in mammalian cells with respect to the effector domain. However, TALEs binding the transcribed region appear to inhibit transcription, possibly via blocking the transcriptional machinery. Intriguingly, in the mammalian system, TALEs appear to have strand-independent activity when binding the promoter region but strand-dependent activity when binding the transcribed region. Taken together, these findings may serve as starting point for a deeper understanding of TALE-mediated gene regulation in the mammalian system.

Conflict of interest

BL and EC declare financial competing interest as SBI (System Biosciences) employees. CUS declares competing interest as a collaborator with SBI.

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