

Engineered TAL Effector modulators for the large-scale gain-of-function screening

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ABSTRACT

Recent effective use of TAL Effectors (TALEs) has provided an important approach to the design and synthesis of sequence-specific DNA-binding proteins. However, it is still a challenging task to design and manufacture effective TALE modulators because of the limited knowledge of TALE–DNA interactions. Here we synthesized more than 200 TALE modulators and identified two determining factors of transcription activity *in vivo*: chromatin accessibility and the distance from the transcription start site. The implementation of these modulators in a gain-of-function screen was successfully demonstrated for four cell lines in migration/invasion assays and thus has broad relevance in this field. Furthermore, a novel TALE–TALE modulator was developed to transcriptionally inhibit target genes. Together, these findings underscore the huge potential of these TALE modulators in the study of gene function, reprogramming of cellular behaviors, and even clinical investigation.

INTRODUCTION

In the cells of higher organisms, the regulation of specific activities of transcription factors (TFs) is likely to be the most prominent and efficient way for generating elaborate responses to diverse internal and external stimuli (1). Indeed, the modulation of both innate and engineered TFs constitutes a promising approach to the study of gene function, reprogramming of cellular behaviors, and even the clinical investigation and treatment of disease (2,3). A number of studies have shown that engineered zinc finger proteins (ZFPs) can selectively correct, activate or repress many endogenous genes in cell lines, animals or humans involved with HIV, Hemophilia or Huntington's disease (4–8). More

recently, Khalil and colleagues demonstrated a strategy to engineer complex functions in *Saccharomyces cerevisiae* by constructing eukaryotic transcription functions using artificial ZFPs (9).

Alternatively, TAL Effectors (TALEs) have become another important modulator to the generation of sequence-specific DNA-binding proteins due to their unique components and functional features (10–12). TALEs comprise a variable number of tandem repeats (often 16 or more) in order to guarantee their DNA-binding specificity and affinity. One repeat binds a single nuclear base, and the region known as 'repeat variable diresidues' has been associated with the specificity of nucleotide binding (such as HD = C, NG = T, NI = A and NN = G or A) (13,14). Thus, for any given DNA sequence, it is easier to design or synthesize a TALE that specifically binds to. Importantly, several platforms have been developed to facilitate the large-scale synthesis of TALEs (15–19).

TALEs fused with VP16/64 were previously shown to bind a specific site in the upstream promoter region of target genes and recruit the transcription complex to initiate gene transcription (15,17,20). However, it is still a challenging task to design and manufacture effective TALE modulators, since our understanding of the molecular mechanism underlying TALE–DNA interactions is at present extremely limited. To address this challenge, we investigated the effect of genome structure on the endogenous transcription activity of TALEs, and demonstrated the feasibility of the large-scale implementation of TALE-VPs in the gain-of-function screen of functional genes regulating cell migration or invasion.

MATERIALS AND METHODS

Cell culture, siRNA and primers

The cell lines HeLa, 293T, A549, MCF-7 and HCT 116 were cultured in Dulbecco's Modified Eagle Medium containing 10% FBS, 100 units/ml penicillin and 0.1 mg/ml strepto-

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mycin under humidified conditions in 95% air and 5% CO₂ at 37°C.

Human kinase siRNA duplexes were obtained from the Silence[®] Select Human Kinase siRNA Library (Ambion). The silence efficiency of these RNAi constructs for their targeted mRNAs was quantitatively tested by real-time polymerase chain reaction (PCR) at 24–48 h after transfection. All reactions were assayed at least in triplicate. The sequences of the primers used in real-time PCR are listed in Supplementary Table S1.

Construction of a TALE-VP or TALE-TALE repressor

The microbeads-based synthesis of TALE has been previously described (17). Briefly, biotinylated DNA tags (200 ng) were immobilized on streptavidin-coated magnetic beads (1 μl) obtained from Invitrogen (DynaL MyOne T1) following the standard protocol provided by the manufacturer. After 15 min, the beads were washed with 1X BW buffer 3× and then digested with SpeI or NheI restriction enzymes (NEB) in a total volume of 30 μl at 37°C for 1 h. A ligation reaction on microbeads was then performed with T4 DNA ligase (NEB) in a total volume of 30 μl at room temperature for 1 h.

The next repeat unit was ligated to the beads in a step-wise fashion, following the same protocol. The final ligation products were then released by SpeI from the microbeads and purified by electrophoresis and gel extraction (Biomed). The extracted TALE products were cloned into pCS2 plasmids as described previously (19). After amplification in *Escherichia coli*, plasmids were then extracted and sequenced. Target-TALE-VP16 or NC-TALE-VP16 was then transfected into HeLa or 293T cells using Lipofectamine 2000 (Invitrogen). After 48 h, images were captured by charge-coupled device (CCD) camera under fluorescence microscopy or cells were quantitatively analyzed by FACS. The sequence of the TALE scaffold, the form of VP16 and the linker between TALE domains are provided in Supplementary Table S2.

Migration assays by a Self-assembly Cell Microarray (SAM-Cell)

Images for Self-assembly Cell Microarray (SAMCell) were taken with a Nikon TE 2000-E (CCD: Regita 2000R, Qimaging, Canada). Briefly, an array pattern of poly-N-isopropylacrylamide was microfabricated through oxygen plasma etching on a glass slide. Then, each TALE-VP16 solution was printed on the slide according to the polymer pattern. We next added culture media mixed with cells on a Petri dish containing a TALE-VP16-printed slide and incubated the dish at 37°C for 1–2 days. After polymer removal, an array of cell islands was formed, each being reverse-transfected with one type of TALE-VP16 plasmid. By calculating the island area change as function of time, we could determine the influence of each TALE-VP16 on cell migration. Areas of cell islands were measured using Image J software. Four repeats were performed in each assay. Since hundreds of different siRNAs or TALE vectors are tested by the migration chip, the Benjamini and Hochberg false discovery rate were used as a correction for multiple tests with

a *P*-value cut-off of 0.05 using JMP 9.0 software. We only selected siRNA or TALE candidates that had an effect over 20% and *P*-value cut-off under 0.05.

Transwell migration and invasion assays

For migration assays, cells were seeded into the upper chamber of a Transwell insert (pore size, 8 μm; Costar) in 100 μl serum-free medium per well. Then, 600 μl medium containing 10% serum was placed in the lower chamber to act as a chemoattractant. Nonmigratory cells were removed from the upper chamber by scraping the membrane. The cells remaining on the lower surface of the insert were fixed with 4% formaldehyde (Sigma) and stained by DAPI (Roche). For invasion assays, cells were seeded in a Matrigel (Bio-Rad)-coated chamber and were incubated at 37°C.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) experiments were performed in duplicate on chromatin isolated from 1 × 10⁸ HeLa or 293T cells using an anti-histone H3 antibody (Abcam, ab1791) following the protocol essentially as previously described (21). Cells were cross-linked with 1% formaldehyde at room temperature for 15 min and DNA was analyzed by PCR-electrophoresis. The sequences of the primers used in ChIP-PCR are listed in Supplementary Table S3.

Immunoblotting

Lysates were resolved by electrophoresis, transferred to a polyvinylidene difluoride membrane (PVDF, Millipore Corporation), and probed with antibodies against PAK2 (CST) or GAPDH (Santa Cruz). The antibodies were used at a 1:1000 dilution.

Luciferase assays

For the luciferase assay, 4.0 × 10⁴ HeLa cells were co-transfected with 200 ng of the indicated pGL3 firefly luciferase construct and 20 ng of a pGL3 Renilla luciferase normalization control. The indicated reporter plasmid or TALE-VPs were also transfected. The medium was changed 6 h post-transfection and luciferase activity was measured after 48 h using the dual luciferase reporter assay system (Promega).

Statistical analysis

For statistical analysis, two-sided unpaired Student's *t*-tests were processed in Excel software. A *P*-value < 0.05 was considered statistically significant: * indicated *P* < 0.05, ** indicated *P* < 0.01 and *** indicated *P* < 0.001. Error bars represented standard deviations (SDs) of at least three independent experiments, unless otherwise indicated. *P*-values are provided, but in general, *P* < 0.05 was considered to be statistically significant.

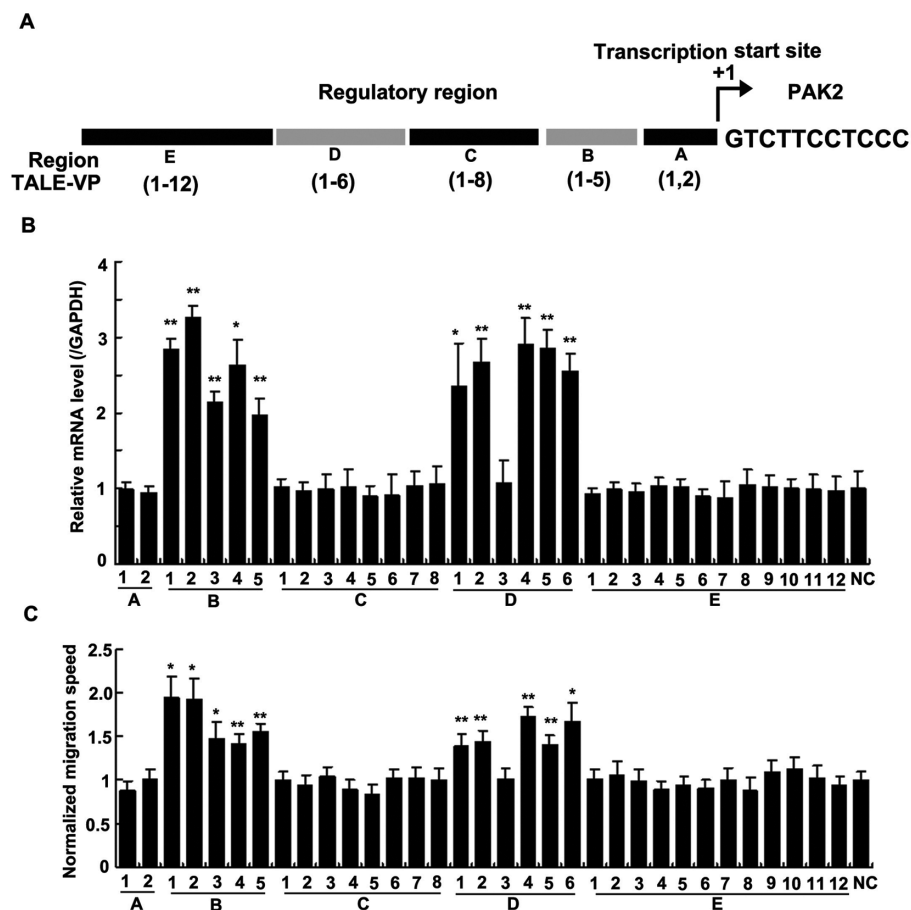


Figure 1. Expression and functional analysis of the activity of TALE-VP16s as well as the regulatory sequence of the PAK2 gene. (A) Schematic diagram showing the regulatory region and transcription start site of the PAK2 gene. The regulatory region is divided into five regions: A-E. In each region, a group of TALE-VPs were designed; (B) Real-time PCR results showing the pattern of expression activation of TALE-VP16s in HeLa cells. (C) SAMCell migration assay showing the capacity of TALE-VP16s to regulate the migration of HeLa cells. * indicates $P < 0.05$; ** indicates $P < 0.01$ (t -test). The error bars represent the SDs of at least three independent experiments.

RESULTS

Construction of TALE-VPs with endogenous transcription activity

We manufactured a series of TALE-VP16s, each specifically bound to a given DNA sequence located before the transcription start site of the PAK2 gene (Figure 1A, Supplementary Figure S1). We first investigated the endogenous transcription activity of these TALE-VP16s in HeLa cells. Strikingly, a wave-like pattern of alternating peaks and valleys was observed on basis of real-time PCR. The targeting sequence was categorized into five regions, i.e. fragments A to E, based on the activity (Figure 1A, Supplementary Figure S1). The TALE-VPs targeting region B or D demonstrated significant transcription activity, whereas those targeting region A, C or E showed no activity.

We then assessed whether the above-mentioned TALE-VP16s had the functional activity through a transwell migration assay. PAK2 is one member of a family of serine/threonine kinases that is involved in the regulation of cytoskeleton regulation and cell motility, and an increase in PAK2 expression promotes cell migration. Consistently, the

migratory regulation curve demonstrated exactly the same wave-like pattern (Figure 1B).

In order to characterize the molecular mechanism underlying the wave-like patterns observed for the TALE-VPs, we first investigated their binding affinity to the target regions of A, C, or E *in vitro* (Figure 2A). We cloned and inserted each region fragment into the promoter region of a normal pGL3 vector, which was referred to as a pGL3-X reporter (X stands for any choice from A to E). Each reporter was transfected with the indicated TALE-VP into HeLa cells. All TALE-VPs tested considerably enhanced the luciferase activity of the corresponding reporters relative to a NC TALE-VP or a control TALE-VP (Figure 2A, Supplementary Figure S2A). For example, the pGL3-A reporter was activated by TALE-VP A1 or A2, but not by TALE-VP B3. These results indicated that the TALE-VPs tested per se have the ability to bind the DNA regions.

We then investigated the molecular mechanism underlying the failure of most TALE-VPs bound to regions A, C or E to activate the transcriptional expression of the PAK2 gene *in vivo*. We hypothesized that the accessibility of a DNA sequence could be affected by the occupancy of RNA polymerase/TFs or histones, which would affect its binding

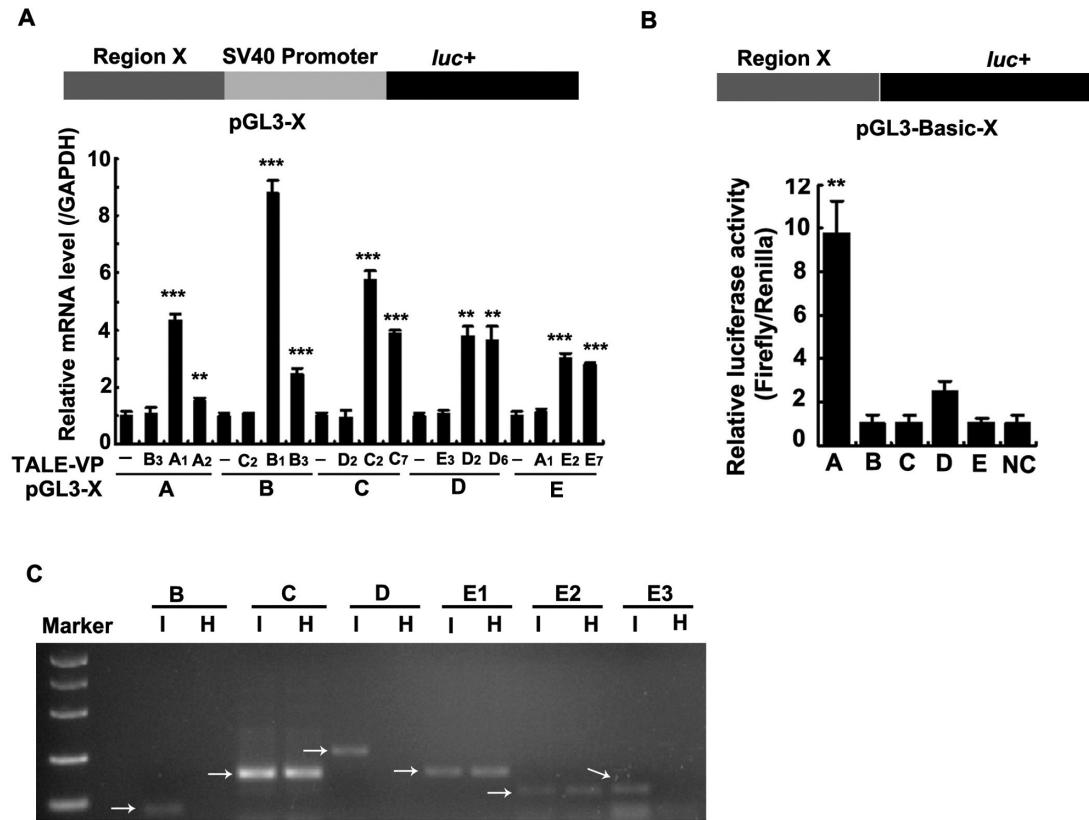


Figure 2. Two main factors affect the activity of TALE-VP16s *in vivo*. (A) Luciferase activity after the co-transfection of a group of indicated TALE-VPs with the corresponding pGL3-X reporters. X stands for region A, B, C, D or E. A negative control TALE-VP was tested in each group. For example, in the case of pGL3-A, the B3 TALE-VP was used as a negative control. (B) Luciferase activity from the co-transfection of a group of indicated TALE-VPs with the corresponding pGL3-basic-X reporters. X stands for region A, B, C, D or E. (C) Chromatin Immunoprecipitation (ChIP) PCR assay showing the status of each indicated fragment bound to the immunoprecipitated histone in HeLa cells. The white arrows indicate the PCR bands after the ChIP-PCR assay. I: input, H: immunoprecipitated histone. * indicates $P < 0.05$; ** indicates $P < 0.01$, *** indicates $P < 0.001$ (*t*-test). The error bars represent the SDs of at least three independent experiments.

affinity to a TALE *in vivo*. In order to identify the region to which RNA polymerase/TFs bound, we constructed another group of reporters by cloning each region fragment into a pGL3-basic vector and referred to as 'pGL3-basic-X'. The pGL3-basic vector lacks any eukaryotic promoter and enhancer sequences and cannot produce luciferase protein. However, when a reporter was transfected into HeLa cells, the original promoter of PAK2 that can be activated by endogenous RNA polymerase/TFs would activate the expression of luciferase protein. We found that only the reporter containing fragment A induced the luciferase activity, whereas fragments B, C, D and E did not (Figure 2B). Both DNaseI hypersensitivity analysis and Txn factor ChIP assay showed that strong signals could be found in region A according to the data from the UCSC Genome Browser (22,23). These results indicated that fragment A contains the core promoter region of PAK2, which inhibits the binding of TALE-VP (A1 or A2).

We next assessed the effect of chromosomal structure on the transcription activity of TALE-VP16. Chromatin Immunoprecipitation followed by PCR was performed to identify the DNA region wrapped around histones. Since region E is longer than 600 bp, we arbitrarily divided it into E1, E2 and E3. In HeLa cells, we found that region C, E1

and E2 were associated with histones, whereas regions B and D as well as E3 were not (Figure 2C, Supplementary Figure S2B). We observed the same results in 293T cells. Thus, chromosome structure plays a determining role in the interaction between a TALE-VP and its targeting DNA sequence.

To confirm that the findings for the PAK2 gene could be applied to other genes, we next synthesized an additional two groups of TALE-VP16s that each specifically bound to a given DNA sequence before the transcription start site of the HK1 or ERBB2 genes. For each gene, we observed the similar wave-like patterns of transcription activation (Figure 3A and B or Supplementary Figures S3 and S4), cell migration activity *in vivo* (Figure 3C and D) and chromosome patterns (Figure 3D and E). Taken together, these results indicate that: (i) the accessibility of regulatory DNA sequence is the main factor for determining the activation of TALE-VPs *in vivo* and (ii) the target DNA fragment must not be located far from the promoter region (e.g. region E3 or F) and is preferably within 1.5 kb in order to effectively activate the expression.

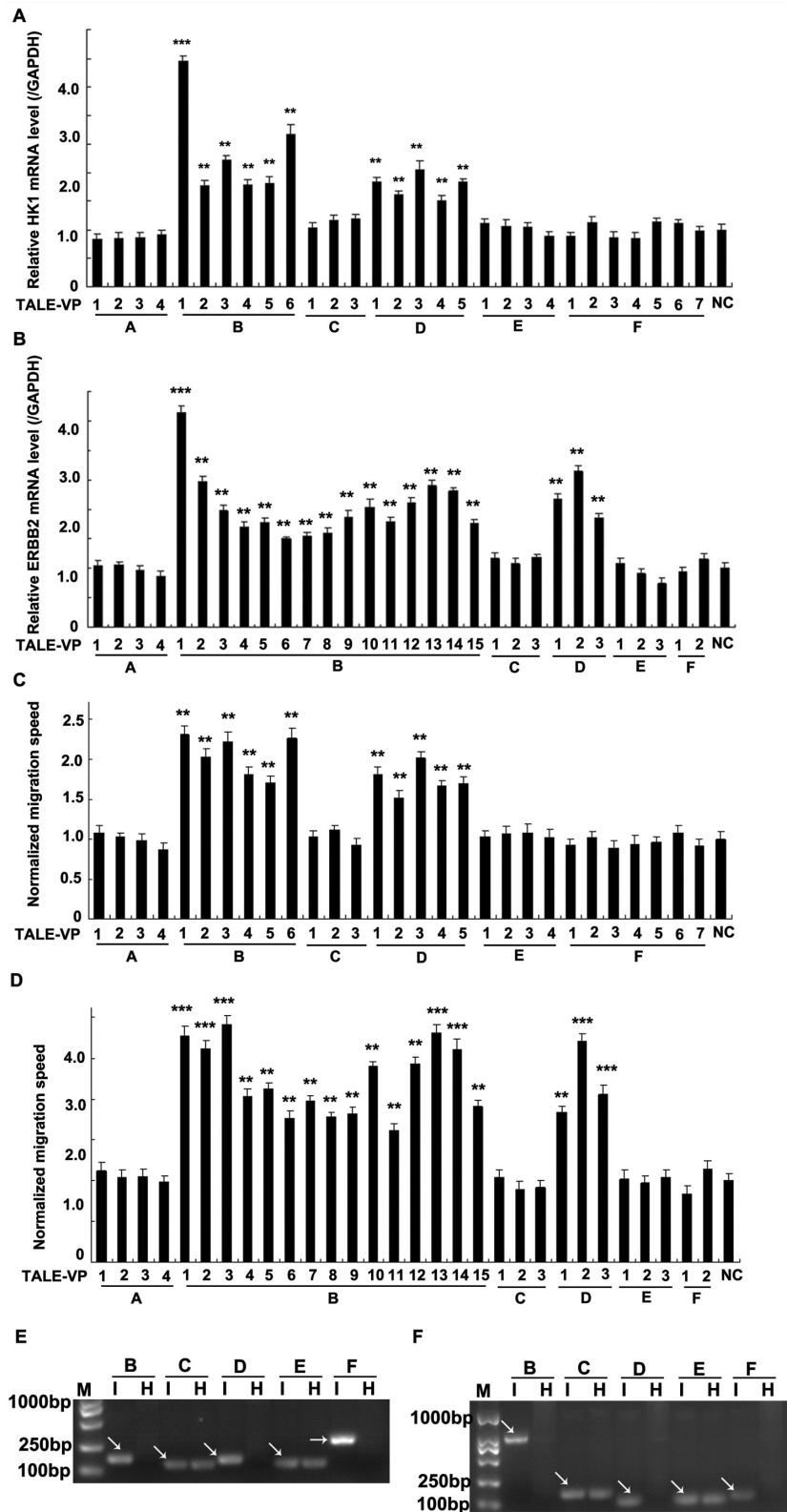


Figure 3. Expression and functional analysis of the activity of TALE-VP16s along the regulatory sequence of the HK1 or ERBB2 genes. (A, B) Real-time PCR results showing the pattern of expression activation of TALE-VP16s activating HK1 (A) or ERBB2 (B) in HeLa cells; (C, D) Transwell migration assay showing the capacity of TALE-VP16s activating HK1 (C) or ERBB2 (D) to regulate the migration of HeLa cells. (E, F) Chromatin Immunoprecipitation (ChIP) PCR assay showing the status of each indicated fragment bound to the immunoprecipitated histone in HeLa cells. The immunoprecipitated pattern of the HK1 gene is shown in E while the pattern for the ERBB2 gene is shown in F. The white arrows indicate the PCR bands after ChIP-PCR assay. I: input, H: immunoprecipitated histone. * indicates $P < 0.05$; ** indicates $P < 0.01$, *** indicates $P < 0.001$ (t -test). The error bars represent the SDs of at least three independent experiments.

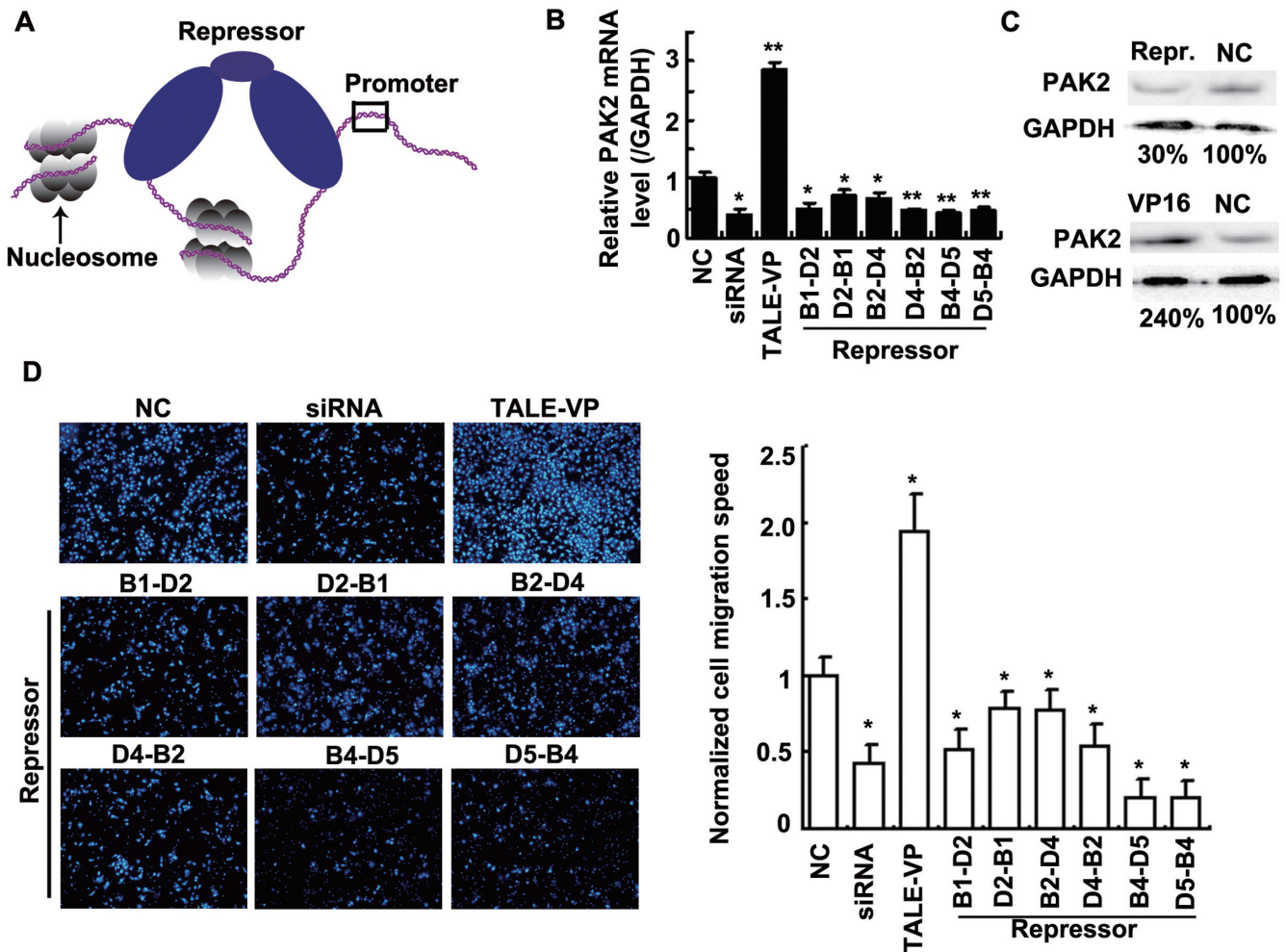


Figure 4. Expression and functional regulation by TALE–TALE repressors. (A) Schematic diagram showing the mechanism of gene repression by a TALE–TALE repressor. (B) Real-time PCR results showing the expression levels after transfection with the indicated TALE–TALE repressor in HeLa cells. siRNA and TALE-VP B1 were used as controls. (C) Western blot results showing the translation levels after transfection with the TALEB1-D2 repressor (Repr.) or TALE-VP B1 in HeLa cells. (D) Transwell migration assay showing the capacity of the indicated TALE–TALE repressors to regulate the migration of HeLa cells. siRNA and TALE-VP B1 were used as the controls. * indicates $P < 0.05$; ** indicates $P < 0.01$ (t -test). The error bars represent the SDs of at least three independent experiments.

TALE–TALE repressor

We then developed a novel type of TALE repressor based on the hypothesis that the conjugation of two TALEs having a strong binding affinity to the neighboring DNA regions would block the binding of RNA polymerase II or TFs, thus inducing steric repression of transcriptional expression (Figure 4A). A series of TALE–TALE repressors were constructed by conjugating TALE B1 and D2 (B1-D2 or D2-B1), B2 and D4 (B2-D4 or D4-B2), or B4 and D5 (B4-D5 or D5-B4). The quantitative analysis based on real-time PCR showed that these repressors, similar to siRNA, significantly repressed the expression of *PAK2* in HeLa cells (Figure 4B). Similar findings were observed in 293T cells (Supplementary Figure S5). Furthermore, western blot assay indicated that these repressors reduced the expression level of *PAK2* to 30%, while TALE-VP16 increased the protein level by more than 2-fold (Figure 4C). We further examined whether these repressors had an effect on the func-

tion of the genes by using a migration assay. Data from the transwell assays demonstrated that these repressors inhibited the migration of HeLa cells similar to siRNA (Figure 4D).

Large-scale gain-of-function screen of kinase genes regulating cell migration or invasion

We performed a large-scale implementation of TALE-VPs into a cell migration assay using the previously reported SAMCell (24). Over 80 TALE-VP16s targeting a subclass of kinase genes were synthesized and functionally characterized. For comparison, the corresponding siRNAs were synthesized and assayed in parallel. We found that all of the TALE-VPs demonstrated exactly the opposite effect on cell migration as their corresponding siRNAs (Figure 5A). In order to examine the activation specificity of TALE-VPs, we randomly selected two genes and performed rescue experiments. We found that HK1 or AKT1 siRNAs could reverse

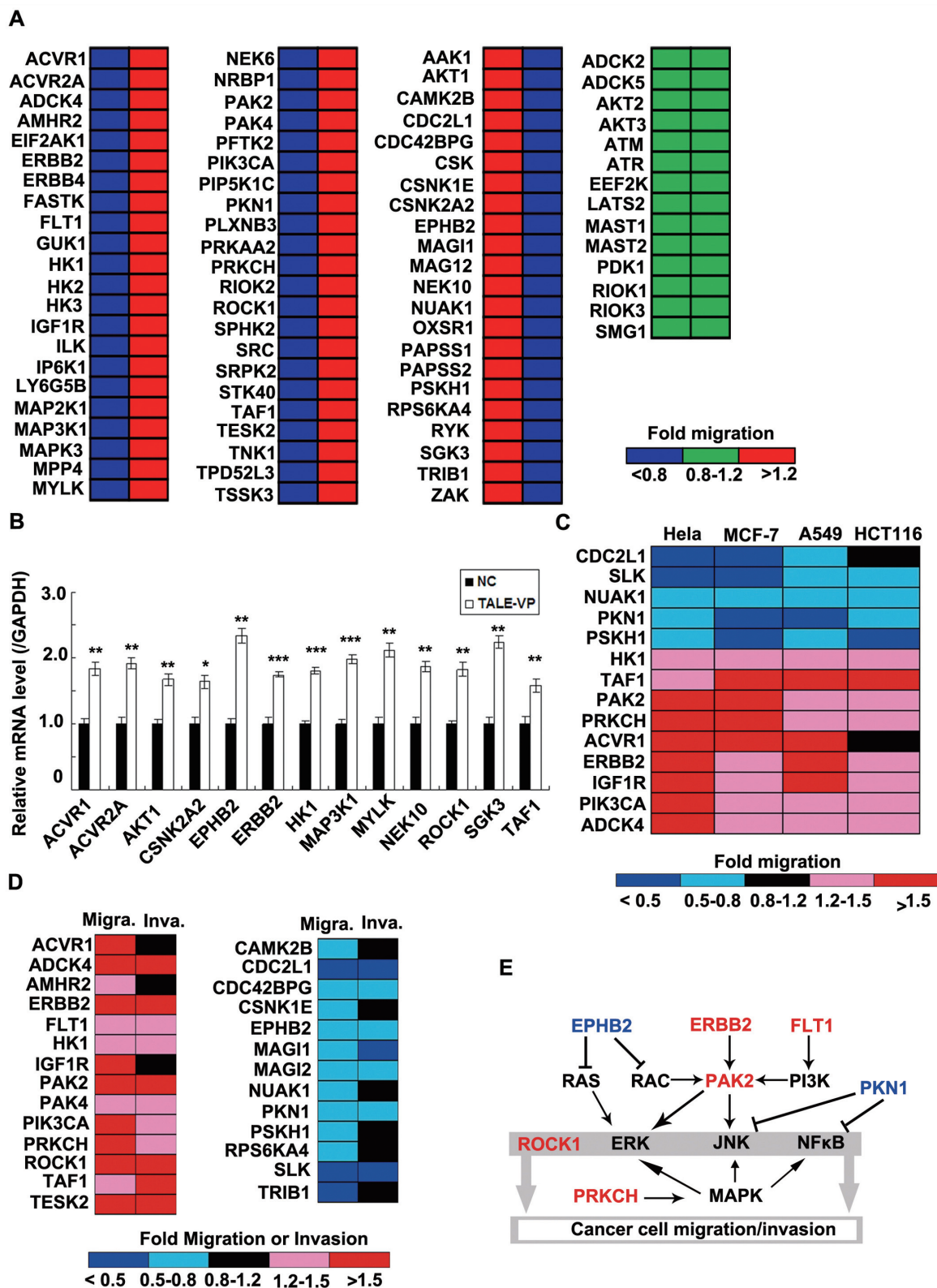


Figure 5. Gain-of-function analysis of the capacity of TALE-VPs to regulate cell migration or invasion. (A) Heat maps showing the capacity of TALE-VPs (right column) or their corresponding siRNAs (left column) to regulate migration in HeLa cells. (B) Real-time PCR results showing the expression levels after transfection with the indicated TALE-VP16s in HeLa cells. (C) General regulatory effect of TALE-VPs on migratory behaviors. Noticeably, no TALE-VPs exhibited a contrasting effect on migratory capacity. (D) Heat maps showing the capacity of TALE-VPs to regulate migration (Migra.) and invasion (Inva.) in HeLa cells. (E) Gene ontology analysis showing that numerous kinase genes, including ERBB2, EPHB2, FLT1, PAK2, PRKCH and ROCK1, participate in certain critical signaling pathways, including the ERK, JNK and NFκB pathways. * indicates $P < 0.05$; ** indicates $P < 0.01$ (t -test). The error bars represent the SDs of at least three independent experiments.

the effect of the corresponding TALE-VPs (Supplementary Figure S6). In addition, we examined the transcription activity of over a dozen randomly selected TALE-VPs and all of them exhibited a >2-fold enhancement at the transcription level (Figure 5B). We also investigated the false negative rate of the gain-of-function screen and found of fourteen genes, each TALE-VP demonstrated negligible capability to regulate cell migration relative to the NC TALE-VP (Figure 5A). These results indicated that the gain-of-function screen based on TALE-VPs has a low false negative rate (Figure 5A).

We next investigated whether these screening results are applicable to other cell types. Over a dozen TALE-VPs were randomly selected and delivered into MCF-7, A4549 or HCT 116 cells. We found that almost all of them exhibited the same regulatory capacity in the three cell lines as in HeLa cells, with the exception of a few TALE-VPs that did not have any obvious impact (Figure 5C).

Finally, we assessed the TALE-VPs in cell invasion assays and found that two-thirds of them were capable of regulating cancer cell invasion (Figure 5D). Gene ontology analysis showed that dozens of kinase genes, including ERBB2, EPHB2, FLT1, PAK2, PRKCH and ROCK1, participate in certain critical signaling pathways, such as the ERK, JNK and NF κ B pathways (Figure 5E). Taken together, our results demonstrated that TALE-VP is an efficient and robust gain-of-function screening approach.

DISCUSSION

To date, a number of techniques for modulating gene expression have been applied in both basic research and clinical studies, such as RNA interference, antisense RNA and ribozymes. RNAi technology has been proven to be an efficient and robust tool for loss-of-function studies. However, there has been no effective and convenient method for gain-of-function studies, with the exception of introducing cDNA libraries. In comparison, TALE modulators have the advantage of exerting not only a repressive effect, but also an activating effect, depending on the fused functional domains. The large-scale TALE-assembly technologies reported recently allow for the manufacture of hundreds of TALE-VPs within a short period of time. In this study, we systematically investigated the factors affecting the binding of TALE-VPs to the promoter region *in vivo* and found two main determinants: the accessibility of the regulatory area and the preferred distance from the transcription start site. We believe that the findings reported in this work may be applicable to other TF-based modulator-DNA interactions.

A few TALE-based repressors have been reported in the literature that are fused with a repressor domain (25). Here, we report a novel TALE-TALE repressor through the conjugation of two TALEs. The binding of TALE-TALE to two DNA regions that neighbor the promoter would form a large complex of protein/DNA, thus blocking the attachment of RNA polymerase/TF to the promoter. In the future, we will continue to engineer a TALE repressor with a reversibly controllable function.

These TALE modulators have been successfully applied in migration and invasion assays. To integrate SAM-Cell with engineered TALE modulators, we developed a

new platform for a large-scale gain-of-function screen. All TALE-VPs studied here successfully demonstrated the capacity to regulate expression and consequently to modulate migration or invasion. A number of studies as well as our findings have shown that the Rho/ROCK and ERK pathways are essential for cell migration and play a critical role in cancer development (26). Rock1, ERBB2, PAK2, FLT1 or PRKCH can promote cell migration, whereas EPHB2 and PKN1 inhibit cell migration. A few key modulators, such as ERBB2 (HER2), are strongly associated with cancer metastasis and a poor prognosis, and have been proven to be effective drug targets (27). The combination of both loss-of-function and gain-of-function screens allow for the large scale identification of more functional genes involved in cancer cell migration and other behaviors. In summary, we strongly believe that TALE-based TFs have extensive applications in gain-of-function screens, *in vivo* gene manipulation and even clinical investigation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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