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## Synergistic and tunable human gene activation by combinations of synthetic transcription factors

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### Abstract

Mammalian genes are regulated by the cooperative and synergistic actions of many transcription factors. In this study we recapitulate this complex regulation in human cells by targeting endogenous gene promoters, including regions of closed chromatin upstream of silenced genes, with combinations of engineered transcription activator–like effectors (TALEs). These combinations of TALE transcription factors induced substantial gene activation and allowed tuning of gene expression levels that will broadly enable synthetic biology, gene therapy and biotechnology.

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Synthetic biology aims to study the control of gene expression by constructing gene regulation systems from the “bottom-up” in order to better understand natural biological systems and develop useful tools for biotechnology.<sup>1</sup> Despite many significant accomplishments, this field has largely been limited to studying artificial promoter-

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### Author Contributions

P.P., A.M.F., G.E.C., A.J.H. and C.A.G. designed experiments. P.P., D.G.O, J.M.B., A.M.F. and K.A.G. performed the experiments. P.P., F.G., G.E.C., A.J.H. and C.A.G. analyzed the data. P.P. and C.A.G. wrote the manuscript.

transgene systems with one or two transactivators, typically in microorganisms.<sup>1-6</sup> In contrast, the natural regulation of mammalian gene expression is extraordinarily complex and is typically achieved by the combinatorial control of each gene by many regulatory factors. This level of complexity has not yet been achieved in synthetic gene regulation systems and has not been possible for the regulation of endogenous genes. However, the recent emergence of technologies for engineering transcription activator-like effectors (TALEs) targeted to almost any DNA sequence<sup>7-14</sup> provides a unique opportunity for recapitulating this natural complexity. In the current study, the combinatorial regulation of endogenous mammalian genes in their natural chromosomal context is achieved by engineering several TALE transcription factors (TALE-TFs) to bind nearby sites upstream of the transcriptional start site (TSS) for a target gene. The composition of these combinations of independent TALE-TFs can be manipulated to control gene activation. Synergistic regulation of gene expression by multiple transcriptional activators is known to occur via simultaneous binding and stabilization of components of the pre-initiation complex.<sup>15-17</sup> Building on this model, we activated endogenous genes with combinations of engineered transcription factors and were able to tune gene expression levels by systematically varying these combinations.

Each TALE-TF has two distinct protein domains that carry out individual molecular functions: (i) the repeat variable diresidue region binds to DNA at user-specified sequences,<sup>7, 8</sup> and (ii) the VP64 effector domain recruits basal transcriptional machinery<sup>9, 10</sup> (Fig. 1a). This design permits rapid construction of synthetic transcription factors that function as autonomous units.<sup>9, 11, 12</sup> Several TALE-TFs have recently been reported to regulate native mammalian gene expression.<sup>9, 10, 14, 18-21</sup> However, the levels of gene activation in these studies were modest and several genes could not be induced (Supplementary Table 1). Therefore there is clear need for improvements to gene activation strategies that capitalize on the synthetic TALE-TF technology.

We designed several TALE-TFs targeted to the promoter regions of the *IL1RN*, *KLK3* (also known as prostate-specific antigen (PSA)), *CEACAM5* (also known as CEA), and *ERBB2* genes that are implicated in immunomodulation, inflammation, and cancer (Fig. 1b, Supplementary Fig. 1). TALE-TF expression plasmids were transfected into HEK293T cells and TALE-TF expression was confirmed by Western blot (Supplementary Fig. 2). TALE-TF activity was first measured in reporter assays in which luciferase is under the control of the respective gene promoter (Fig. 1c-f). Most individual TALE-TFs activated the co-transfected plasmid reporters, but only at modest levels similar to previous reports (Supplementary Table 1).<sup>9, 10, 14, 18-21</sup> However, the delivery of combinations of TALE-TFs led to substantial synergistic effects on gene activation. Importantly, the synergistic activation of the plasmid-based reporters was recapitulated in upregulation of the native genes in their natural chromosomal context as determined by quantitative RT-PCR, including induction of mRNA levels greater than 10,000-fold (Fig. 1g-j). Detection of induced protein expression of IL-1ra, encoded by the *IL1RN* gene, KLK3, CEACAM5, and erbB-2 by ELISA and Western blot validated the functional outcome of the activation of these genes (Fig. 1k-n). In particular, we only reproducibly detected expression of IL-1ra, KLK3 and CEACAM5 protein in samples with combinations of TALE-TFs. We found low

expression of *ERBB-2* in control samples and cells transfected with single TALE-TFs, but its expression was substantially enhanced in cells transfected with all TALE-TFs (Fig. 1n).

These results are consistent with a mechanism in which the VP64 acidic activation domain of multiple transcription factors is simultaneously interacting with and stabilizing components of the pre-initiation complex.<sup>15-17</sup> This mechanism was confirmed by demonstrating that the VP64 domain was essential to achieving the synergistic effect, indicating that the synergy is not the result of nucleosome displacement by TALEs (Supplementary Fig. 3). The synergistic gene activation was also conserved when using alternative acidic activation domains (Supplementary Fig. 4). The expression of other genes nearby *ILIRN* did not increase, indicating that this large synergistic activation was specific to the target gene (Supplementary Fig. 5).

The TALE-TFs used in this study were not specifically designed to target DNase-hypersensitive regions (Supplementary Fig. 6), in contrast to many other reports of synthetic transcription factors that only target open chromatin. In fact, *ILIRN*, *KLK3*, and *CEACAM5* are not expressed in HEK293T cells. Interestingly, targeting chromatin inaccessible to DNase did not prevent gene activation by the engineered TALE-TFs (Fig. 1g-i). These results suggest that targeting open chromatin may not be a pre-requisite to successful TALE-TF engineering and that activation of silenced genes is possible in the absence of chromatin modifying drugs,<sup>18</sup> particularly when using combinations of TALE-TFs. In contrast to these three genes, *ERBB2* is moderately expressed in HEK293 cells and the TALE-TFs for *ERBB2* regulation were targeted to open chromatin (Supplementary Fig. 6). Combinations of these TALE-TFs also led to synergistic *ERBB2* activation although the effect was not as substantial relative to the other genes as a result of higher levels of basal expression (Fig. 1f,j,n).

To comprehensively characterize the effects of combinatorial regulation of mammalian genes by engineered TALE-TFs, all 63 permutations of six TALE-TFs targeting three different genes were co-transfected with a corresponding luciferase reporter in HEK293T cells (Fig. 2a-c). Various combinations of TALE-TFs could be used to reproducibly achieve tunable levels of gene expression over a large dynamic range. Many TALE-TFs that did not activate the reporter when delivered alone contributed to synergistic activation of expression when combined with other TALE-TFs (Supplementary Table 2). In some cases, the addition of a TALE-TF decreased gene expression. However, for all three genes there was an increase in the average level of gene expression with increased number of TALE-TFs (Fig. 2d), and the average contribution of each additional TALE-TF decreased as the number of TALE-TFs increased (Fig. 2e).

In order to assign quantitative parameters to the relative contribution of each TALE-TF to the synergistic effect across the 63 data points in these experiments, a polynomial model was applied to the data set of each gene of the form

$$y_j = \left( \sum_{i=1}^6 w_i x_{i,j} \right)^2$$

where  $y_j$  is the relative luciferase activity for the  $j^{\text{th}}$  combination of the six TALE-TFs. The value of  $x_{i,j}$  is 0 if the  $i^{\text{th}}$  TALE-TF is not included in the  $j^{\text{th}}$  combination and it is 1 if it is included. The effect coefficient  $w_i$  is a fit parameter that represents the relative contribution of the  $i^{\text{th}}$  TALE-TF to the regulation of its target promoter in the context of all permutations of the six TALE-TFs. Multiple regression was used to solve for values of  $w_i$  for all TALEs for each of the three target genes. These coefficients generate an excellent fit of the experimental data (Fig. 2f-h) and are highly significant ( $P < 2E10^{-3}$ ) in accurately describing the relative contribution of each TALE (Supplementary Table 3). Importantly, the polynomial model provides a stronger description of the data than the corresponding additive and multiplicative models (Supplementary Fig. 7). This is because the additive model does not account for synergy of TALE-TF activity (Fig. 2d) and the multiplicative model does not account for the diminishing contribution of each additional TALE-TF (Fig. 2e). The superior fit of the polynomial model relative to the additive model can be mathematically explained by the second-order terms that are the product of effect coefficients for different TALE-TFs. This suggests the presence of some form of cooperativity, but cannot reveal the underlying mechanism. As discussed above, the simultaneous binding and stabilization of components of the pre-initiation complex by VP64 is likely to play a role,<sup>15-17</sup> as well as other secondary effects of VP64-mediated gene activation on local epigenetics and chromatin structure.

Previous studies have suggested that TALE-TF activity may correspond to proximity to the transcription start site (TSS)<sup>18</sup> or TALE RVD composition.<sup>13</sup> In this study, there was no clear correlation of effect coefficient with TALE array length, composition, or distance to TSS that was consistent for all genes (Supplementary Fig. 1). This suggests that these TALE design parameters cannot independently be used to predict highly effective TALE-TFs. It is likely that other biological and structural components of these gene promoters, including genome folding and competition with endogenous regulatory factors, play a dominant role in determining the activity of single TALE-TFs and TALE-TF combinations.

The cooperative activation of gene expression described here presents a unique opportunity to develop tunable transcription networks that operate at different levels as a function of the number and identity of TALE-TFs. This facilitates the control of gene expression levels without the need for small molecules used in conventional chemically regulated systems. Unlike prior work in synthetic biology that has focused on the regulation of transgenes by engineered promoters customized with multiple transcription factor binding sites<sup>1-6</sup> or gene repression or silencing,<sup>22, 23</sup> the use of TALE-TF combinations that target endogenous promoters begins to recapitulate the complexity of natural systems in a precise and controlled manner. This approach constitutes a powerful experimental system for elucidating fundamental mechanisms of natural gene regulation that are currently poorly understood. The capacity for combinatorial regulation also provides a novel framework for engineering biocomputation systems that control endogenous genes in mammalian cells, similar to recently developed genetic logic gates that control engineered transgenes.<sup>2-6</sup> Precise control of gene expression with multiple tunable inputs may lead to greater robustness and predictability in bioengineered systems in the context of cell-machine interfaces and gene- and cell-based therapies. For example, this could include increasing the potency of

therapeutic effects of engineered transcription factors.<sup>24</sup> In summary, this approach to gene regulation extends the capacity of synthetic biology and biological programming in mammalian systems and provides a new facile technology for regulation of native mammalian genes with widespread potential applications.

## Methods

### Cell culture and transfection

HEK293T cells were obtained from the American Tissue Collection Center (ATCC) through the Duke University Cancer Center Facilities and were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO<sub>2</sub>. HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Transfection efficiencies were routinely higher than 95% as determined by flow cytometry following delivery of a control eGFP expression plasmid. The amount of DNA used for lipofection was 800 ng per well in 24-well plates or 200 ng per well in 96-well plates. For luciferase reporter assays in 24-well plates, 100 ng of reporter plasmid was included with 700 ng of TALE-TF expression plasmid. When comparing single TALE-TFs to the combination of all TALE-TFs (Fig. 1), the total amount of TALE-TF expression plasmid was held constant (i.e. 800 ng of single TALE-TFs vs. 800 ng of total TALE-TF expression plasmid divided equally amongst each factor). When assessing the individual contribution of each TALE-TF (Fig. 2), the amount of each TALE-TF was held constant at 116 ng, with empty expression plasmid added to a total of 700 ng. Amounts of DNA for transfections in 96-well plates were scaled accordingly.

### Plasmids and TALE assembly

TAL effectors were assembled using the Golden Gate TALEN and TAL effector kit obtained from Addgene.<sup>11</sup> A destination vector for the final assembly step was created to include a Flag epitope tag and an SV40 NLS at the N terminus, a 152 residue deletion from the N terminus of the *wt* TALE proteins that has previously been shown to preserve the DNA binding ability of TALEs,<sup>10</sup> 63 *wt* TAL amino acids following the repeat domain,<sup>9</sup> a C-terminal SV40 NLS, a VP64 domain that contains 4 repeats of the minimal activation domain of VP16, and an HA tag at the C terminus (Fig. 1a). TAL effectors were originally designed to target within the 600 bp upstream of the transcriptional start site (Fig. 1b) based on the criteria described by Cermak et al.,<sup>11</sup> although a subsequent study showed that these guidelines did not affect TALE functionality.<sup>12</sup> TALEs were designed downstream of the transcriptional start site for *ERBB2*, but upstream of the translation start site, based on previous studies showing high levels of activity of synthetic zinc finger transcription factors targeting this region.<sup>25</sup> The compositions of all TALE-TFs used in this study are provided in Supplementary Fig. 1.

The reporter plasmids were built by cloning PCR-amplified genomic DNA sequences upstream of the genes of interest *ILIRN* (chromosome 2, 113874366-113875462), *ERBB2* (chromosome 17, 37855857-37856492), *CEACAM5* (chromosome 19, 42211804-42212651) and *KLK3* (chromosome 19, 51357466-51358177) into the vector pGL3-Basic (Promega). Coordinates are provided based on the hg19 reference genome.

## Luciferase assays

Forty-eight hours after transfection, cells were collected into 96-well plates, washed with PBS once and lysed with 100 mM monobasic sodium phosphate and 0.2% Triton X-100. The lysate was incubated with Bright-Glo™ Substrate (Promega) in a 1:1 ratio and luciferase activity was measured using a Synergy 2 Multi-Mode Microplate Reader (BioTek). The results are expressed as relative luciferase activity, which is the average luciferase activity normalized to luciferase activity in samples transfected with the reporter vector and empty TALE-TF expression vector. Data is presented from at least three independent experiments performed with 2 technical replicates per experiment.

## Western blot

Cells were lysed in 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100 and 0.1% SDS. Protein concentrations in cell lysates were measured by the BCA Protein Assay (Pierce). Lysates were mixed with loading buffer, boiled for 5 min, and equal amounts of protein were run in NuPAGE® Novex 4-12% Bis-Tris Gel polyacrylamide gels and transferred to nitrocellulose membranes. Non-specific antibody binding was blocked with 50 mM Tris/150 mM NaCl/0.1% Tween-20 (TBS-T) with 5% nonfat milk for 30 min. The membranes were incubated with primary antibodies (HRP-conjugated anti-HA (Roche, clone 3F10) in 5% milk in TBS-T diluted 1:5000 for 30 min; anti-CEACAM5 (Cell Signaling Technology, clone CB30) in 5% milk in TBS-T diluted 1:1000 overnight; anti-GAPDH (Cell Signaling Technology, clone 14C10) in 5% milk in TBS-T diluted 1:5000 for 30 min; anti-ERBB2 (Cell Signaling Technology, clone 29D8) in 5% BSA in TBS-T diluted 1:2000 for 2 h) and the membranes were washed with TBS-T for 30 min. Membranes labeled with primary antibodies were incubated with anti-rabbit HRP-conjugated antibody (Sigma-Aldrich, cat. no. A6154) diluted 1:5000 for 30 min, and washed with TBS-T for 30 minutes. Membranes were visualized using the Immun-Star WesternC™ Chemiluminescence Kit (Bio-Rad) and images were captured using a ChemiDoc™ XRS+ System and processed using ImageLab software (Bio-Rad).

## ELISA

Serum-free culture media (OPTI-MEM) was collected and frozen at -80°C. Human IL-1ra and KLK3 secretion into culture media was quantified via enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocols (R&D Systems, Cat. No. DY280 and DKK300, respectively). For the IL-1Ra ELISA, the standard curve was prepared by diluting recombinant human IL-1ra in OPTI-MEM and the IL-1ra in culture media was measured undiluted. For the KLK3 ELISA, the standard curve was prepared by diluting recombinant KLK3 in the manufacturer's calibrator diluent. The samples were concentrated ~8 fold via centrifugation through 3 kDa MWCO filters for 20 minutes (Amicon Ultra, Cat # UFC500396). Reported values were corrected by the concentration factor for each sample.

For both assays, optical density was measured at 450 nm with a wavelength correction at 540 nm. Each standard and sample was assayed in duplicate. The duplicate readings were averaged and normalized by subtracting the average zero standard optical density. A standard curve was generated by log transforming the data and performing a linear regression of the IL-1ra or KLK3 concentration versus the optical density. The lower limit

of detection was 50 pg/ml for human IL-1ra and 32 pg/ml for human KLK3. Data reported are the mean and s.e.m. of these individual values combined from multiple experiments (n = 6 biological replicates for IL-1ra, n = 4 biological replicates for KLK3).

### Quantitative RT-PCR

Total RNA was isolated using the RNeasy Plus RNA isolation kit (Qiagen). cDNA synthesis was performed using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). Real-time PCR using SsoFast™ EvaGreen® Supermix (Bio-Rad) was performed with the CFX96 Real-Time PCR Detection System (Bio-Rad) with 45 cycles, melting for 2 s at 95°C, and annealing and extension for 2 s at 55°C. Real-time PCR oligonucleotide primers ((ERBB2 5'-AGCCGCGAGCACCCAAGT-3', 5'-TTGGTGGGCAGGTAGGTGAGTT-3'), CEACAM5 (5'-TCCCCACAGATGGTGCAT-3', 5'-GAACGGCGTGGATTCAATAG-3'), KLK3 (5'-CTCGTGGCAGGGCAGTCT-3', 5'-AGCTGTGGCTGACCTGAAAT-3'), IL1RN (5'-GACCCTCTGGGAGAAAATCC-3', 5'-GTCCTTGCAAGTATCCAGCA-3') PSD4 (5'-GCAGCACCTCCTGGTCAC-3', 5'-ATCCGACACATCCTGATTCC-3'), IL1F10 (5'-CCTCCCCATGGCAAGATACT-3', 5'-AGCAGTTGTCTGCAACAGGA-3') and GAPDH (5'-CAATGACCCCTTCATTGACC-3', 5'-TTGATTTTGGAGGGATCTCG-3')) were designed using Primer3Plus software and purchased from IDT. Primer specificity was confirmed by agarose gel electrophoresis and melting curve analysis. Reaction efficiencies over the appropriate dynamic range were calculated to ensure linearity of the standard curve.

### Statistics

Statistical analysis was performed by single factor ANOVA with alpha equal to 0.05 in Microsoft Office Excel 2007. Effect coefficients were determined using the Regression tool in the Data Analysis Add-In to Microsoft Office Excel 2007, with the Relative Luciferase Activities (Fig. 2a-c, Supplementary Table 2) serving as the y input and an array of zeros and ones representing each TALE-TF combination as the x input.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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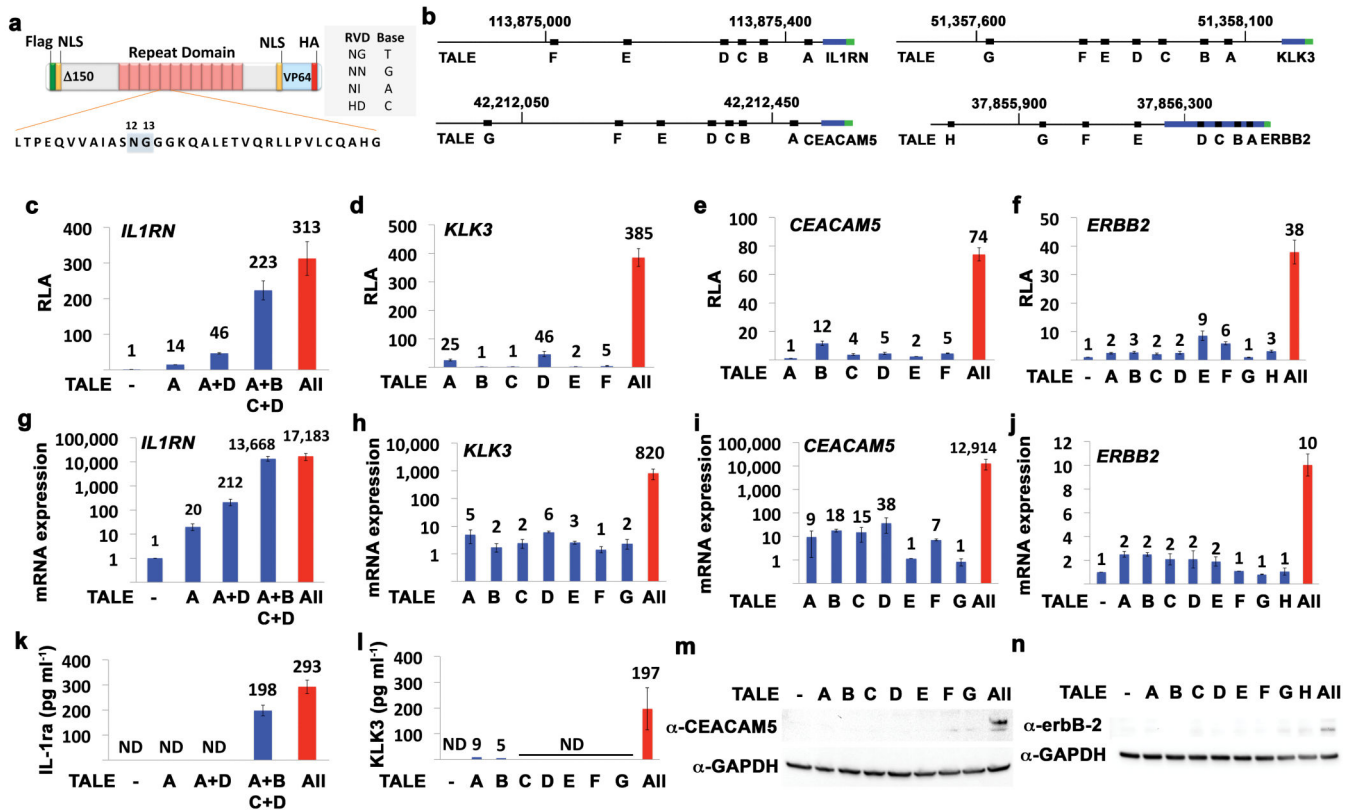
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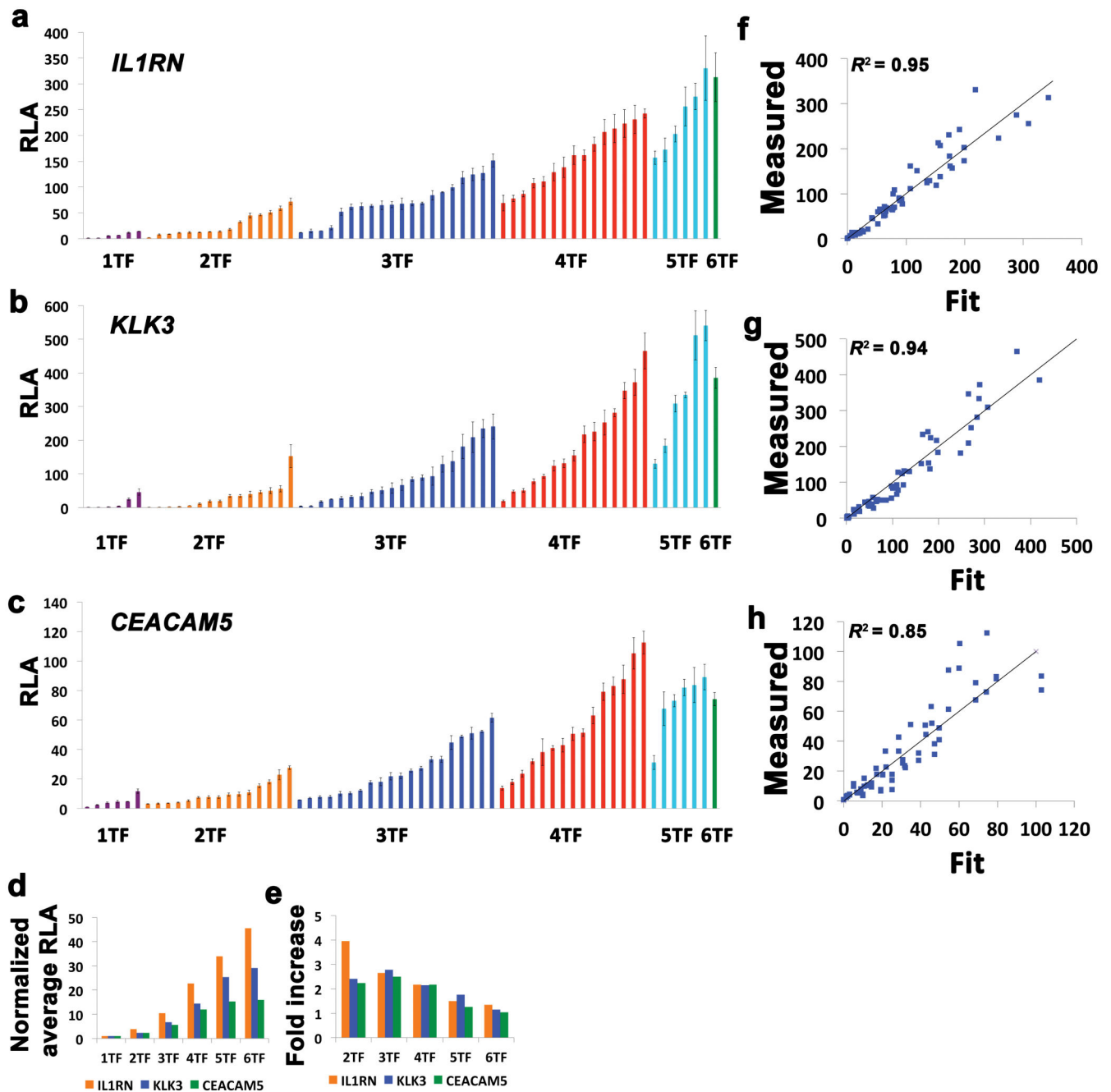
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**Figure 1.**

Synergistic activation of gene expression by combinations of TALE-TFs. (a) Structure and sequence of TALE-TFs in this study. (b) Genomic positions of TALE-TF target sites in the *CEACAM5*, *KLK3*, *IL1RN*, and *ERBB2* genes (hg19 coordinates) are indicated by black boxes. Transcribed and coding regions are shown in blue and green, respectively. (c-f) Relative luciferase activity (RLA) in promoter reporter assays. (g-j) Relative mRNA expression levels measured by quantitative RT-PCR and (k-n) protein expression levels assayed by ELISA or Western blot for each target gene in human cells transfected with the indicated TALE-TFs. Each gene is organized by column.  $n = 3$  unless indicated otherwise in the online methods. Mean  $\pm$  SEM and  $P < 0.0001$  by ANOVA for all bar graphs.



**Figure 2.**

Combinatorial regulation of gene expression by TALE-TFs. (a-c) All possible 63 combinations of six TALE-TFs targeting the *IL1RN*, *KLK3*, and *CEACAM5* genes were tested for activation of a luciferase reporter plasmid and ordered according to number of TALE-TFs and magnitude of relative luciferase activity (RLA). Samples receiving the same number of TALE-TFs are colored identically. Mean  $\pm$  SEM ( $n = 3$ ) and  $P < 0.0001$  by ANOVA for all three data sets. (d) The average RLA for the indicated number of TALE-TFs is presented for each gene. (e) The fold increase of RLA for each number of TALE-TFs

relative to the average RLA for one less TALE-TF is presented for each gene. (f-h) The measured values for all 63 combinations of TALE-TFs are plotted versus the values fit by the polynomial model along with  $y = x$ . (i) Effect coefficients and corresponding  $P$ -values resulting from multiple regression of the polynomial model are provided for each TALE-TF.