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Howard Hughes Medical Institute

Published as: *Nat Methods*. 2013 August ; 10(8): 762–767.

TALE-mediated modulation of transcriptional enhancers

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Abstract

We tested whether Transcription Activator-Like Effectors (TALEs) can mediate repression and activation of endogenous enhancers in the *Drosophila* genome. TALE-repressors (TALERs) targeting each of the five *even-skipped* (*eve*) "stripe" enhancers generated repression specifically of the focal stripes. TALE-activators (TALEAs) targeting the *eve* promoter or *eve* enhancers caused increased expression primarily in cells normally activated by the promoter or targeted enhancer, respectfully. The phenotypic effects of TALER and TALEA expression in larvae and adults are consistent with the observed modulations of *eve* expression. In these assays, the *Hairy* repression domain did not exhibit previously described long-range transcriptional repression activity. The precise effects of the TALEAs support the view that repression acts in a dominant fashion on transcriptional activators and that the activity state of an enhancer influences TALE binding or the ability of VP16 to enhance transcription. TALEs thus provide a novel tool for detection and functional modulation of transcriptional enhancers in their native genomic context.

Transcriptional enhancers encode patterns of gene expression by binding transcription factor proteins that recognize specific sequences within enhancers and enhancers often integrate the combined activity of multiple transcription factors[1]. Transcriptional enhancers can be located close to or up to hundreds of kilobase pairs from their respective gene promoters[1]. Alteration in enhancers underlie development, evolution, and disease[1] and, in many eukaryotic genomes, more DNA may encode transcriptional enhancers than encodes proteins[2]. Despite the importance of transcriptional enhancers, we currently understand far less about the structure and function of enhancer regions than we do about protein coding regions.

Our understanding of enhancer structure and function is derived mainly from reporter gene assays, wherein putative enhancer DNA is coupled to a heterologous promoter and reporter. Reporter gene assays have provided most of our current knowledge of enhancer structure and function. These studies indicate that transcriptional regulation of some, but not all, eukaryotic genes is modulated by multiple enhancers that act independently[3]. Despite the

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Author Contributions
JC conceived of, designed, and executed the experiments and analyzed the data, with mentorship of DLS. JC and DLS wrote the manuscript.

The authors declare they have no competing financial interests.

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insight that has been provided by reporter gene assays, these experiments suffer from several limitations. First, reporter constructs often drive incomplete and/or ectopic patterns of expression[4], probably because enhancers are tested away from their native genomic context. Second, reporter constructs rarely drive expression at normal levels, which confounds quantitative studies of gene regulation. Third, some studies have failed to identify modular autonomous enhancers that recapitulate components of the complete expression pattern[3,5,6]. Publication bias probably has resulted in under-reporting of genes that appear to lack modular enhancers[5].

To provide a method complementary to classical reporter-gene assays, we exploited Transcription Activator-Like Element (TALE) DNA-binding proteins to target transcriptional repressor and activator protein domains to specific genomic locations. TALEs can be engineered to target specific DNA sequences[7,8] and TALE DNA-binding domains fused to activators and repressors and targeted specifically to promoters can modulate gene expression in plants[9,10] and in human cell-culture[11–16]. In the present paper we demonstrate that TALEs can be targeted to enhancers to modulate specific domains of complex expression patterns.

Results

Experimental design

We engineered GAL4 responsive vectors for *Drosophila melanogaster* transgenesis that allow fusion of a TALE DNA-binding domain to regulatory domains[17] (Fig. 1a, Supplementary Fig. 1, and Supplementary Text 1). In each of these fusion genes the native activator domain of the TALE C-terminus was removed. We tested the Krüppel and Hairy repression domains in TALE-mediated repressors (TALERs) and the VP64 (four tandem copies of VP16) activation domain in TALE-mediated activators (TALEAs). Estimates of repressor activity from reporter-gene assays suggest that Krüppel can repress enhancers within approximately 100 bp of a DNA binding site[18], whereas Hairy can reportedly silence enhancers up to 5 kb from a DNA binding site[19,20].

As a proof of principle, we targeted the well-studied enhancers of the gene *even-skipped* (*eve*), which encodes a transcriptional repressor required for correct segmentation and neuronal development[21–23]. *Eve* transcripts appear first in the blastoderm embryo and expression resolves rapidly into seven transverse stripes along the anterior-posterior axis (see Fig. 1). Separate enhancers drive subsets of these stripes (Fig 1b), apparently autonomously[23,24].

TALER mediate repression of the *eve* promoter

To determine the efficiency of TALERs in the embryo, we drove ubiquitous, zygotic expression of a TALER-Hairy targeted near the *eve* promoter (Fig. 1b). This TALER-Hairy reduced expression of all *eve* stripes (Fig. 1) and resulted in abnormal expression of engrailed (*en*), a target of Eve[22] (*cf.* Fig. 1). Larval cuticles of these embryos exhibited fused segments (Fig. 1). To test whether the residual *eve* expression in these embryos resulted from late onset of TALER expression relative to *eve* activation, we drove this TALER-Hairy

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with a maternally-expressed driver[25]. In these embryos, *eve* expression was almost undetectable (Supplementary Fig. 2), *en* expression was disrupted severely (Fig. 1), and outward signs of segmentation in the larval cuticle were lost (Fig. 1). These results are consistent with the effects of *even-skipped* hypomorphic alleles[26]. We also drove this TALER-Hairy using neurogenic GAL4 drivers, and, in all cases, we observed decreased Eve levels in neurons (Supplementary Fig. 3). As a control, a TALE-GFP fusion protein targeted to the same site did not alter *eve* expression (Supplementary Fig. 4). Promoter-targeted TALERs thus provide a complementary tool to existing conditional gene silencing technologies in *Drosophila*[27]. In addition, judicious use of GAL4 drivers may be used to allow TALERs to mimic an allelic series.

TALEA mediated activation of the *eve* promoter

We examined next, whether TALEs could be used to selectively activate gene expression. To confirm the efficiency of the activator fusion, we drove ubiquitous, zygotic expression of a TALEA targeted near the *eve* promoter. Strikingly, these embryos exhibited stronger and broader patterns of expression of all seven stripes of Eve, compared to wild type (Fig. 2). While we observed low levels of Eve expression between the canonical stripes, there is still a clear 7-striped pattern of expression. Engrailed expression was disrupted in these embryos (Fig. 2), as expected[28].

TALER mediated repression of transcriptional enhancers

Given the efficiency of TALE-mediated transcriptional repression, we tested whether TALERs could regulate specific transcriptional enhancers. We generated TALEs that targeted each of the five stripe-specific enhancers and the autoregulatory element of *eve*. It has been hypothesized that the regulatory autonomy of individual enhancers results from the action of short-range repressors, such as *Krüppel*[18]. It is also possible that the genomic context of *eve* enhancers allows enhancers to act independently.

As a partial test of these alternative hypotheses—and to identify the most useful reagents—in separate experiments we drove ubiquitous expression of a TALER-Krüppel and a TALER-Hairy targeted to a 16 bp sequence within the *eve* stripe 2 enhancer[29,30]. Both TALERs repressed *eve* stripe 2 expression specifically, and the TALER-Hairy generated stronger repression than did the TALER-Krüppel (Fig. 3). We observed no notable changes in the levels of expression of other *eve* stripes (Fig. 3), even though the enhancer for stripes three and seven is located only 1.6 kb upstream from the targeted binding site (Fig. 1). These embryos lost a single stripe of *engrailed* expression (Fig. 3), which is consistent with the *engrailed* phenotype produced by a deletion of *eve* stripe 2[30]. Furthermore, these embryos failed to hatch and larval cuticles exhibited an altered gnathal segment (Fig. 3), as expected[26]. As a control, ubiquitous expression of a TALEGFP fusion protein targeted to the same 16 bp sequence in *eve* stripe 2 did not alter *eve* expression (Fig. 3). All together, these results suggest that both Krüppel and Hairy can generate local repression of an enhancer in its native genomic location, although Hairy appears to drive stronger repression than does Krüppel. We therefore used TALER-Hairy fusion proteins for all other repression experiments.

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Ubiquitous expression of TALER-Hairy fusion proteins targeting each of the remaining *eve* stripe enhancers (Fig. 4a) caused reduced expression primarily of those stripes corresponding to the previously reported expression domain of each enhancer (Fig. 4b–d and Supplementary Fig. 5). In multiple cases, TALER-Hairy repressed stripes of *eve* are expressed in fewer cell rows, consistent with previous observations that *eve* enhancers are sensitive to repressor concentrations[31]. A TALER-Hairy targeted to the minimal autoregulatory sequence (MAS), located approximately 5 kb upstream of the *eve* promoter, caused strong reduction in expression of all *eve* stripes after embryonic stage 5, as expected[32] (Supplementary Fig. 6). We found that a TALER-Hairy construct targeting the stripe 4/6 enhancer caused a slight reduction also in *eve* stripe 5 expression (Fig. 4c). However, TALERs targeting two different binding sites within the 4/6 enhancer produced similar patterns of repression of stripes four and six (Supplementary Fig. 5), while only one of these TALERs reduced expression of stripe 5. While this is an interesting observation, we cannot rule out the possibility that repression of stripe 5 by one TALER represents an experimental artifact. Each TALER-Hairy construct generated precise and predicted patterns of disruption of *en* and phenotypic effects in larval cuticles (Supplementary Fig. 7). We observed no evidence for ‘long-range’ repression by the TALER-Hairy constructs, suggesting that, in a native genomic context, Hairy may function at a more limited range, or with greater specificity, than suggested previously[24].

TALEA mediated activation of transcriptional enhancers

The precise spatial and temporal domains of enhancer activity are believed to result, primarily, from the activity of repressors that limit the activity of more broadly expressed activators[31]. While the quantitative level of activators is clearly important for determining the final level of gene expression[33], it is thought that most activators are unable to overcome the limiting effects of repressors[31]. If this is true, then targeting an additional activator to an enhancer should influence gene expression only, or mainly, in an expression domain that is already active. We tested this idea by targeting TALEAs to multiple *eve* enhancers.

Ubiquitously expressed TALEAs targeted to the stripe 3/7, stripe 4/6, and stripe 5 *eve* enhancers each caused an increase in the level of expression specifically in the stripe driven by the native enhancer (Fig. 4e–g). In several cases, the targeted *eve* stripe was expressed in more cell rows than in wild-type embryos. In two cases, TALEAs influenced primarily one stripe of an enhancer that was previously reported to regulate two stripes; the TALEA targeting the stripe 3/7 enhancer mainly increased stripe 3 expression and the TALEA targeting the 4/6 enhancer mainly increased stripe 4 expression (Fig. 4h and 4j). There are several possible explanations for these observations. First, while these composite enhancers cannot be divided cleanly by reporter assays into fragments that drive separate stripes, the regulatory information encoded in these enhancers may be sufficiently spatially segregated that a TALEA can influence mainly one stripe. Alternatively, the VP64 activator may be less efficient at activating some enhancers, depending on interactions with other repressive and activating factors occupying a given enhancer.

Each of the TALEAs we tested resulted in the fusion of *en* stripes that flanked the altered *eve* stripes (Fig. 5a–d). Remarkably, adult flies developed from embryos treated with all

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three TALEAs: TALEA stripe 4/6 adults displayed reduced abdominal segments one and six (compare Fig. 5e and Fig. 5f); TALEA stripe 3/7-adults displayed fusion of the T2 and T3 segments, including loss of a pair of legs, and reduced abdominal segment seven (Fig. 5g); and TALEA stripe 5 adults exhibited a reduced abdominal segment five (Fig. 5h). These results also reinforce that although we observed weak activation of *eve* stripes 7 and 6 (see above), these manipulations were sufficient to disrupt normal development of these body regions.

TALER specificity for a minimal transcriptional enhancer

All together, these observations indicate that ubiquitously expressed TALEs fused to a repressor or an activator and targeted to single regulatory elements can generate specific effects. As a further test of the specificity of the TALEs, we compared the effect of the TALER-Hairy targeted to *eve* stripe 2 on a synthetic *D. melanogaster eve* stripe 2 construct and the homologous *D. pseudoobscura eve* stripe 2 construct, which differs by 3 bp from the *D. melanogaster* construct at the target sequence (Supplementary Fig. 8). When the TALER-Hairy was expressed ubiquitously, we observed lower expression of the *D. melanogaster* reporter gene, but no change in expression of the *D. pseudoobscura* reporter gene[34] (Supplementary Fig. 8), suggesting that this TALE, at least, displays high specificity for its target site.

Discussion

These results indicate that individual regulatory elements in the genome can be targeted *in situ* with single transcriptional repressors or activators using TALEs. We were surprised that a single TALE could provide robust repression and we hypothesize that the protein-DNA interaction for TALEs is more specific than binding observed for metazoan transcription factors, which seem to have evolved relatively low specificity protein-DNA interactions to enable cooperative and synergistic binding[35]. The relatively local effects of the enhancer-TALER-*hairy* constructs that we observed are inconsistent with previous reports of long-range repression by *hairy*[36]. We suggest two hypotheses to explain this discrepancy. First, enhancers may bind proteins—either directly by DNA-protein interactions or indirectly through protein-protein interactions—that prevent interactions between neighboring enhancers. If DNA regions responsible for this hypothetical “antisocial” behavior of enhancers do not promote transcription on their own, then these DNA regions may have been trimmed from “minimal” enhancer fragments that have been used widely in classical reporter gene assays. Second, the DNA between transcriptional enhancers may encode boundary elements that limit the spread of repressor activity. This second hypothesis is consistent with the observation that deleting DNA outside of the “minimal” *eve*S2 leads to decreased transcriptional robustness[30].

Perhaps the most interesting finding is that none of the ubiquitously expressed TALEAs disrupt all seven stripes of *eve* expression or drive expression in other ectopic locations. Even the TALEA targeted to the promoter drives increased expression mainly in the seven-stripe region. There are several distinct possibilities for these results. First, TALEAs may bind to their respective targets in all embryonic cells, but their activating signals may be

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overridden by repressive cues. Alternatively, the TALEA binding sites may be inaccessible to TALEA binding in cells where the enhancers are not normally active. This second hypothesis is consistent with the view that chromatin accessibility is responsible for directing the widespread patterns of *Drosophila* transcription factor binding[37,38].

Our results strongly support a model for combinatorial activation of independent, modular *Drosophila eve* enhancers[4,23,24]. The precise effects of the TALEAs supports the view that repression acts in a dominant fashion on transcriptional activators[31,39]. Because TALERs and TALEAs provide experimental access specifically to active enhancers, they may allow functional dissection of non-modular enhancer architectures that have confounded reporter gene assays.

Methods

Construction of TALE plasmids

TALE constructs were based on the JFRC-7 vector[26] and modified for use with the Golden-Gate method[11] by mutating all Esp3I sites. TALE-C terminus fusion proteins were synthesized by GeneScript and subcloned into JFRC-7 at the XhoI/XbaI sites, removing the mCD8 and GFP domains. The following domains were added in separate constructs: GFP[40]; Kr repression domain, amino acids 402–502; *hairy* repression domain 255–337, VP64 activation domain[40]. Plasmids will be deposited at AddGene (www.addgene.org).

Construction of TALEs

TALE target sites were identified using the TAL Effector-Nucleotide Targeter, TALE-NT[17]. TALEs were subsequently assembled using the Golden-Gate method[17].

Fly Strains and Crosses

Drosophila melanogaster strains were maintained under standard laboratory conditions. Transgenic TALE constructs were created by Rainbow Transgenic Flies Inc., and were integrated at the attP2-landing site. The following GAL4 drivers were used: Actin5C-GAL4; NGT40[25] (Bloomington stock 4442); and rhomboid-GAL4, (Bloomington stock 26871).

Embryo Manipulations

For each respective GAL4 line, virgins were collected and crossed with male, TALE-bearing lines. Embryos were raised at 28°C and collected, and fixed according to standard protocols. Antibody staining was carried out according to standard procedures. Briefly, primary antibodies obtained from the Developmental Studies Hybridoma Bank were used to detect Eve (3C10 used 1:20) and En (4D9 used 1:20) proteins, followed by detection of primary antibodies using secondary antibodies labeled with Alexa Fluor dyes (used 1:500, Invitrogen). Cuticle preps were performed using standard protocols.

Microscopy

Confocal images were obtained on a Leica DM5500 Q Microscope, using an ACS APO 20×/0.60 IMM CORR lens, with Leica Microsystems LAS AP software. Sum projections of confocal stacks were assembled, embryos were scaled to match sizes, background was

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subtracted using 50 pixel rolling ball radius, and plot profiles of fluorescence intensity were analyzed using ImageJ software (rsb.info.nih.gov/ij/). Data from the plot profiles were further analyzed in Matlab.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank T. Martin and A. DePace, Department of Systems Biology, Harvard Medical School, for the *eve*S2-lacZ enhancer flies, E. Preger-Ben Noon and C. Standley for comments on the manuscript, B. Pfeiffer, M. Schroeder, R. Mann and the entire Stern lab for discussion. We thank the anonymous reviews for comments that significantly improved this manuscript. Finally, we thank the Janelia Farm Research Campus community for facilitating this work and for providing an inspiring scientific environment.

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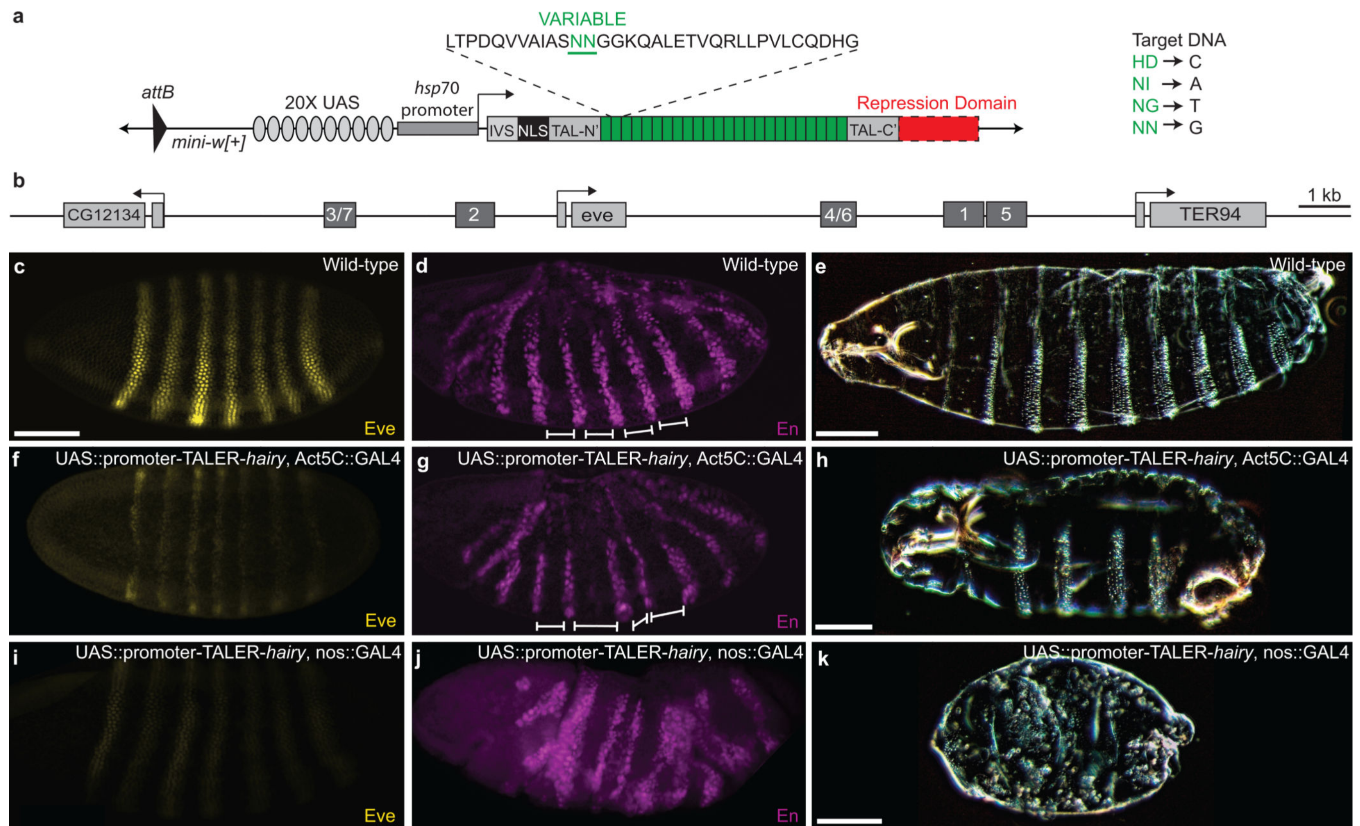

Figure 1.

TALERs targeted to the promoter can repress expression of *even-skipped* (*eve*). (**a**) Schematic of a GAL4 responsive TALER construct. See Supplementary Fig. S1 for the complete list of *Drosophila* TALE constructs. (**b**) Schematic of the *eve* locus, indicating early-embryonic *cis*-regulatory stripe enhancers. (**c, f, i**) Stage-5 embryos stained for Eve protein. (**c**) Wild-type embryos. (**f**) Eve expression is decreased when Act5C::GAL4 drives a UAS::promoter-TALER-*hairy* (*cf.* **f** with **c**). (**i**) Eve expression is further diminished when the UAS::promoter-TALER-*hairy* is driven with a maternal *nos*::GAL4 driver. (**d, g, j**) Stage-11 embryos stained for Engrailed protein. (**d**) Wild-type embryo. (**g**) Stripes of Engrailed expression are variable in width and spacing, noted with white lines, when a Act5C::GAL4 drives the UAS::promoter-TALER-hairy (*cf.* **g** with **d**). (**j**) Stripes of Engrailed are fused and reduced in number when *nos*::GAL4 drives the UAS::promoter-TALER-*hairy*. (**e, h, k**) Larval cuticle preps. (**e**) Wild-type larva. (**h**) UAS::promoter-TALER-*hairy* expression, driven with Act5C::GAL4, causes a reduction in the number of larval segments. (**k**) UAS::promoter-TALER-*hairy* expression, driven with a *nos*::GAL4 driver, causes loss of most or all segmentation. Embryos in panels (**c, d, f, g, i**, and **j**) are matched in scale. Scale bars in (**a, e, h**, and **k**) equal 100 μm.

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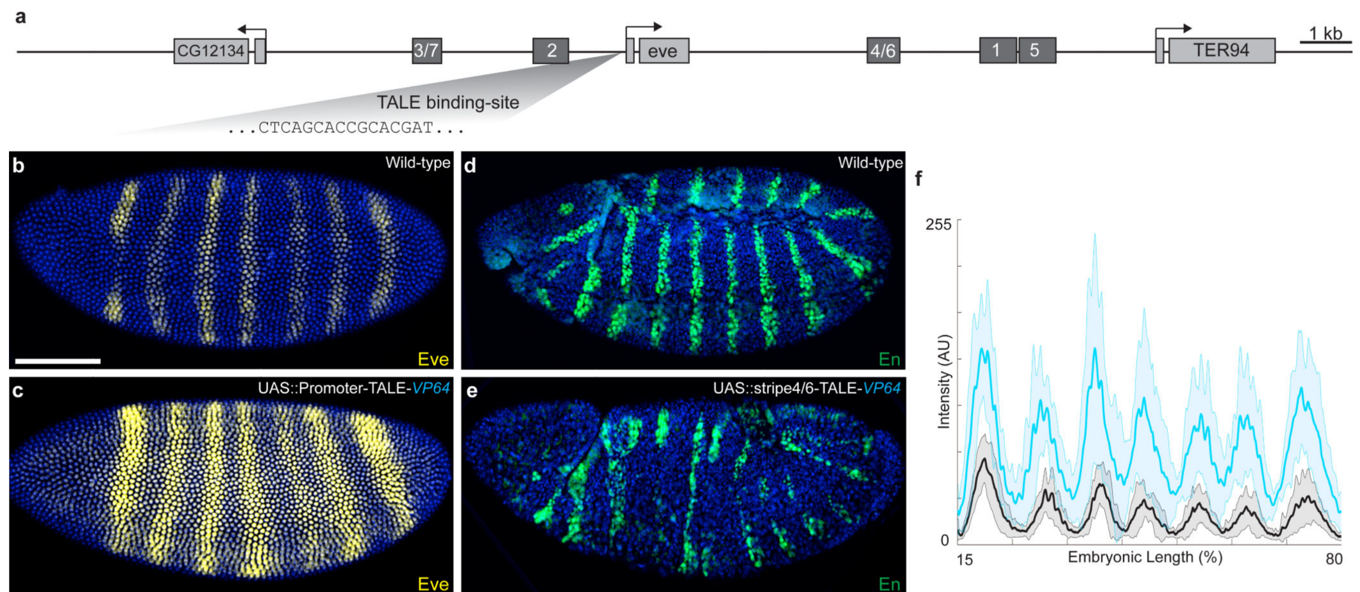

Figure 2.

TALE targeted activation of the *eve* promoter. (**a**) Schematic of the *eve* locus, indicating early-embryonic *cis*-regulatory stripe enhancers and TALE binding site. (**b, c**) Stage-5 wild-type embryo (**b**) and embryo carrying UAS::promoter-TALE-VP64, nos::GAL4 (**c**). (**d, e**) Stage-12 wild-type embryo (**d**) and UAS::promoter-TALE-VP64, nos::GAL4 embryo (**e**), stained for Engrailed (En). (**f**) Profiles of average expression levels of Eve in Stage-5 embryos (n=10 for each genotype). The solid gray line denotes wild-type embryos and the turquois plots denote the promoter-TALER-*VP64*, respectively. Lighter-shaded, bounding areas indicate ± 1 standard deviation. Signal intensity is reported in arbitrary units (AU). Embryos in panels (**b–e**) are matched in scale. Scale-bar in (**b**) equals 100 μm.

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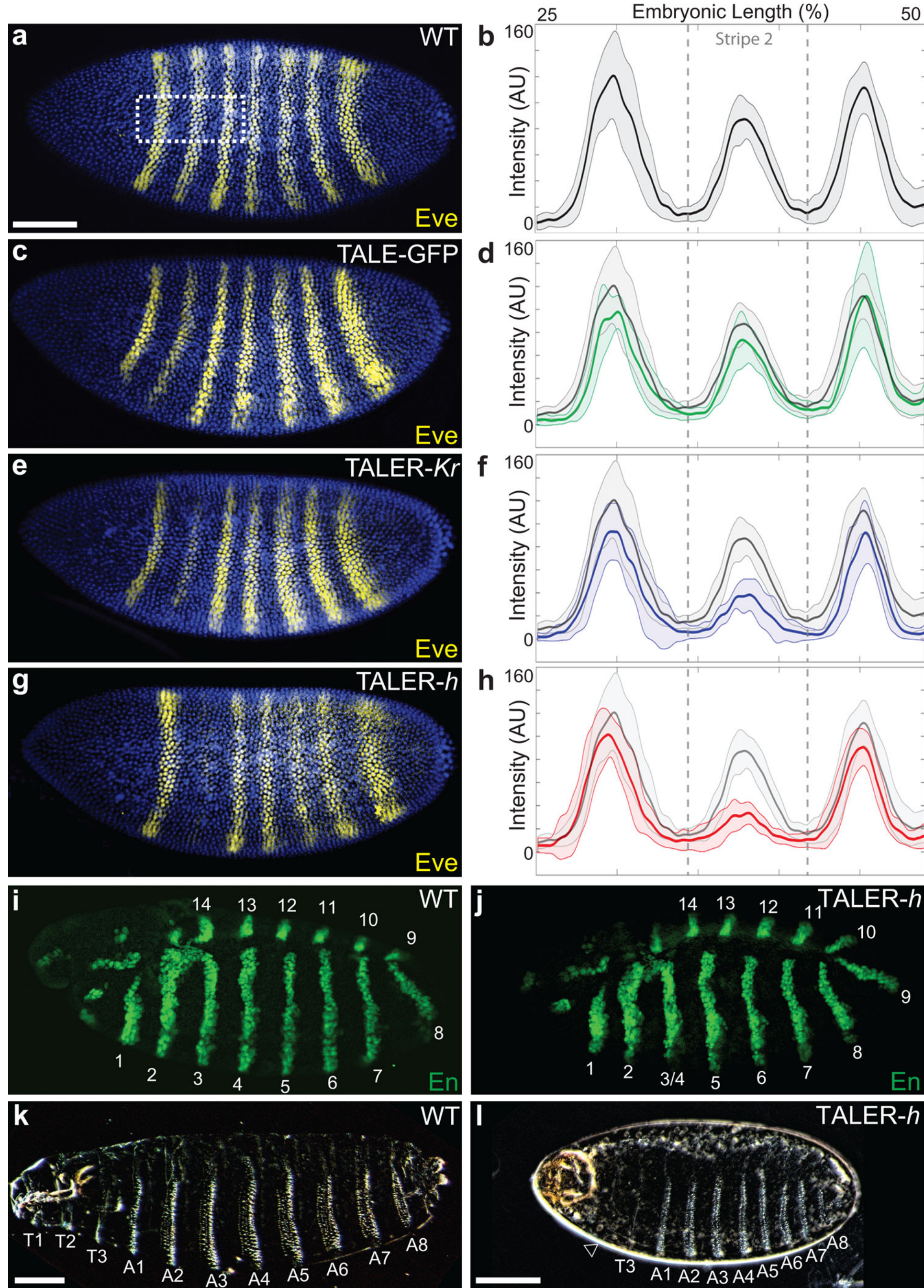

Figure 3.

TALER targeted repression of the *eve* stripe 2 enhancer. (**a, c, e, g**), Stage-5 wild-type embryo (**a**), and embryos carrying UAS::enhancer-TALER-*GFP, nos*::GAL4 (**c**), UAS::enhancer-TALER-*Krüppel, nos*::GAL4 (**e**), or UAS::enhancer-TALER-*hairy, nos*::GAL4 (**g**) stained for Eve protein. (**b, d, f, h**) Profiles of average expression levels in region highlighted in white-dashed box in panel (**a**), of Eve in Stage-5 wild-type embryos (**b**), and embryos carrying UAS::enhancer-TALER-*GFP, nos*::GAL4 (**d**), UAS::enhancer-TALER-*Krüppel, nos*::GAL4 (**f**), or UAS::enhancer-TALER-*hairy, nos*::GAL4 (**h**) (n=10

for each genotype). In all plots, the solid gray line denotes wildtype embryos, with the green (**d**), blue (**f**), and red (**h**) plots denoting enhancer-TALER*GFP*, enhancer-TALER-*Krüppel*, and enhancer-TALER-*hairy*, respectively. Lighter-shaded, bounding areas indicate one standard deviation. AU indicates Arbitrary Units of fluorescence intensity. **i, j**, En protein staining in Stage-12 wild-type embryo (**i**) and Stage-12 UAS::enhancer-TALER-*hairy, nos*::Gal4 embryo (**j**). Parasegments are labeled, with parasegments 3 and 4 fused in the enhancer-TALER-*hairy* embryos. (**k, l**) Cuticle preps of wild-type first instar larva (**k**) and UAS::enhancer-TALER-*hairy, nos*::Gal4 larvae (**l**), with denticle belts labeled. The UAS::enhancer-TALER-*hairy, nos*::Gal4 larva failed to hatch and possessed fused thoracic segments (empty arrowhead). Embryos in panels (**a, c, e, g, i**, and **j**) are matched in scale. Scale bars in (**a, k**, and **l**) equal 100 μm.

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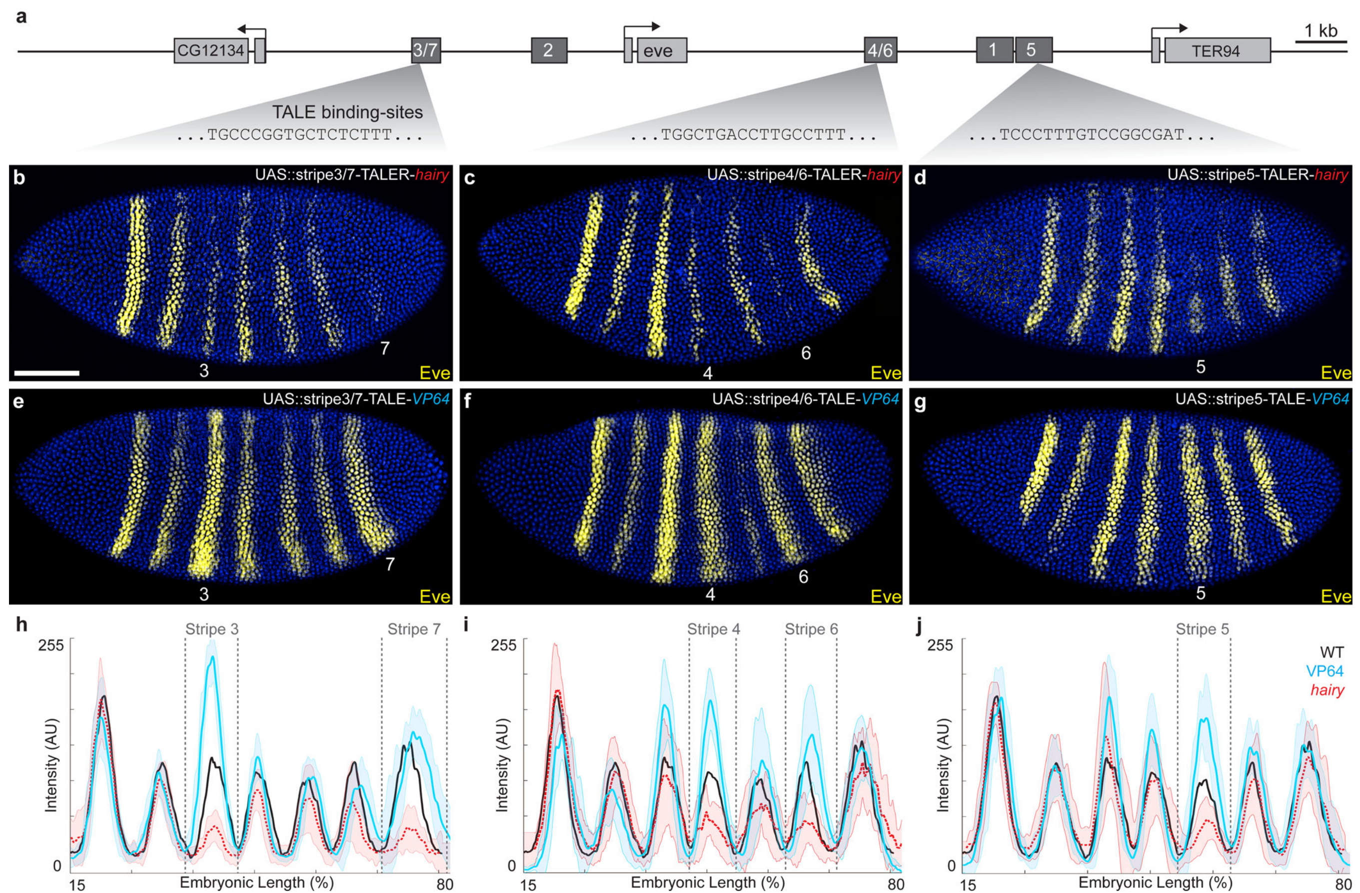

Figure 4.

TALE targeted repression and activation of *eve* stripe enhancers. (**a**) Schematic of the *eve* locus, indicating early-embryonic *cis*-regulatory stripe enhancers and TALE binding sites. (**b–g**) Stage-5 embryos stained for Eve protein and carrying *nos*::GAL4 and either UAS::stripe 3/7-TALER-*hairy* (**b**), UAS:: stripe 4/6 TALER-*hairy* (**c**), UAS::stripe 3/7-TALER-*hairy* (**d**), UAS::stripe 3/7-TALE-*VP64* (**e**), UAS:: stripe 4/6 TALE-*VP64* (**f**), or UAS::stripe 3/7-TALE-*VP64* (**g**). (**h–j**) Profiles of average expression levels of Eve in stage-5 embryos carrying *nos*::GAL4 and either UAS::stripe 3/7-TALE, *nos*::GAL4 (**h**), UAS::stripe 4/6-TALE, *nos*::GAL4 (**i**), UAS::stripe 5-TALE, *nos*::GAL4 (**h**) (n=10 for each genotype). In all plots, the solid gray line denotes wild-type embryos, with the turquois and red-dashed plots denoting enhancer-TALER-*VP64* and enhancer-TALER-*hairy*, respectively. Lighter-shaded, bounding areas indicate one standard deviation. Embryos in panels (**b–g**) are matched in scale. Scale bar in (**b**) equals 100 μm.

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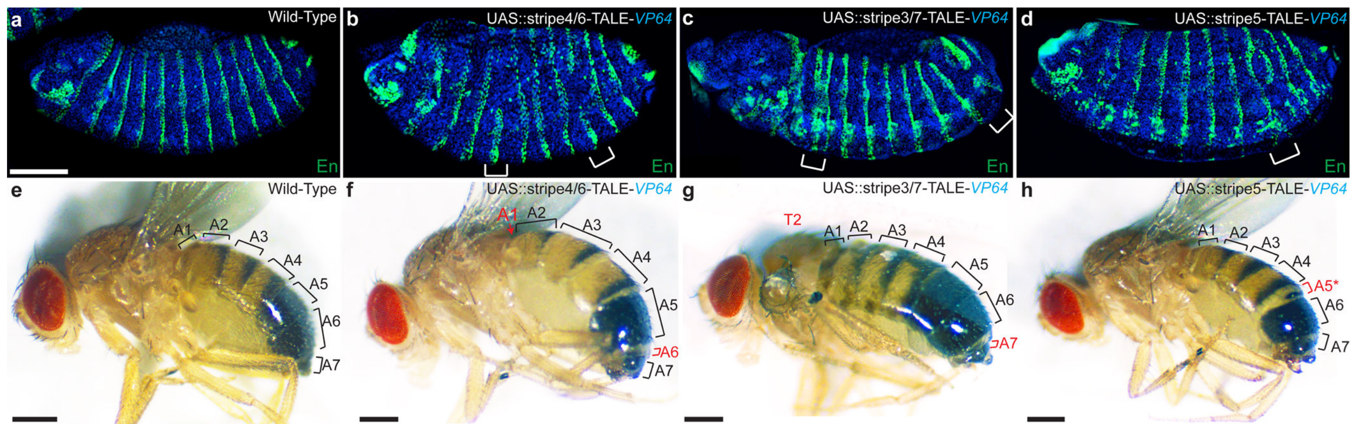

Figure 5.

Phenotypes resulting from enhancer-TALE-*VP64* activation of *eve* enhancers. (**a–d**), Stage-15 wild-type embryo (**a**), and embryos carrying UAS::stripe4/6-TALE-*VP64, nos*::Gal4 (**b**), UAS::stripe3/7-TALE-*VP64, nos*::Gal4 (**c**), or UAS::stripe5-TALE*VP64, nos*::Gal4 (**d**) stained for En protein. Altered and fused parasegments are labeled with white brackets. Embryos in panels (**a–d**) are matched in scale. Scale bar in (**a**) equals 100 μm. (**e–h**), Adult wild type fly (**e**), and adults carrying UAS::stripe4/6-TALE-*VP64, nos*::Gal4 (**f**), UAS::stripe3/7-TALE-*VP64, nos*::Gal4 (**g**), or UAS::stripe5-TALE-*VP64, nos*::Gal4 (**h**). Adult abdominal segments are labeled with fused and altered segments labeled in red. Scale bars in (**e–h**) equal 400 μm.