TALE-mediated modulation of transcriptional enhancers *in vivo*

Justin Crocker & David L Stern

We tested whether transcription activator–like effectors (TALEs) could 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 enhancers caused increased expression primarily in cells normally activated by the promoter or targeted enhancer, respectively. This effect supports 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 the VP16 domain to enhance transcription. In these assays, the Hairy repression domain did not exhibit previously described long-range transcriptional repression activity. The phenotypic effects of TALER and TALEA expression in larvae and adults are consistent with the observed modulations of *eve* expression. 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 in the enhancers, and they 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. These studies indicate that transcriptional regulation of some, but not all, eukaryotic genes is modulated by multiple enhancers that act independently[3]. Despite the 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 remotely 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 used 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 cultured human cells[11–17]. Here we demonstrate that TALEs can be targeted to enhancers to modulate specific domains of complex expression patterns *in vivo*.

RESULTS

Experimental design

We engineered GAL4-responsive vectors for *Drosophila melanogaster* transgenesis that allow fusion of a TALE DNA-binding domain to regulatory domains[18] (**Fig. 1a**, **Supplementary Fig. 1** and **Supplementary Note**). 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 TALERs (pJC-TALE-*Kr* and pJC-TALE-*hairy*) and the VP64 (four tandem copies of VP16) activation domain in TALEAs (pJC-TALE-*VP64*). Estimates of repressor activity from reporter-gene assays suggest that Krüppel can repress enhancers within approximately 100 base pairs (bp) of a DNA-binding site[19], whereas Hairy can reportedly silence enhancers up to 5 kilobase pairs (kb) from a DNA-binding site[20,21].

As a proof of principle, we targeted the well-studied enhancers of the gene *eve*, which encodes a transcriptional repressor required for correct segmentation and neuronal development[22–24]. *eve* transcripts appear first in the blastoderm embryo, and expression resolves rapidly into seven transverse stripes along the anterior-posterior axis (**Fig. 1**). Separate enhancers drive subsets of these stripes (**Fig. 1b**), apparently autonomously[24,25].

Howard Hughes Medical Institute, Janelia Farm Research Campus, Ashburn, Virginia, USA. Correspondence should be addressed to J.C. (crockerj@janelia.hhmi.org).

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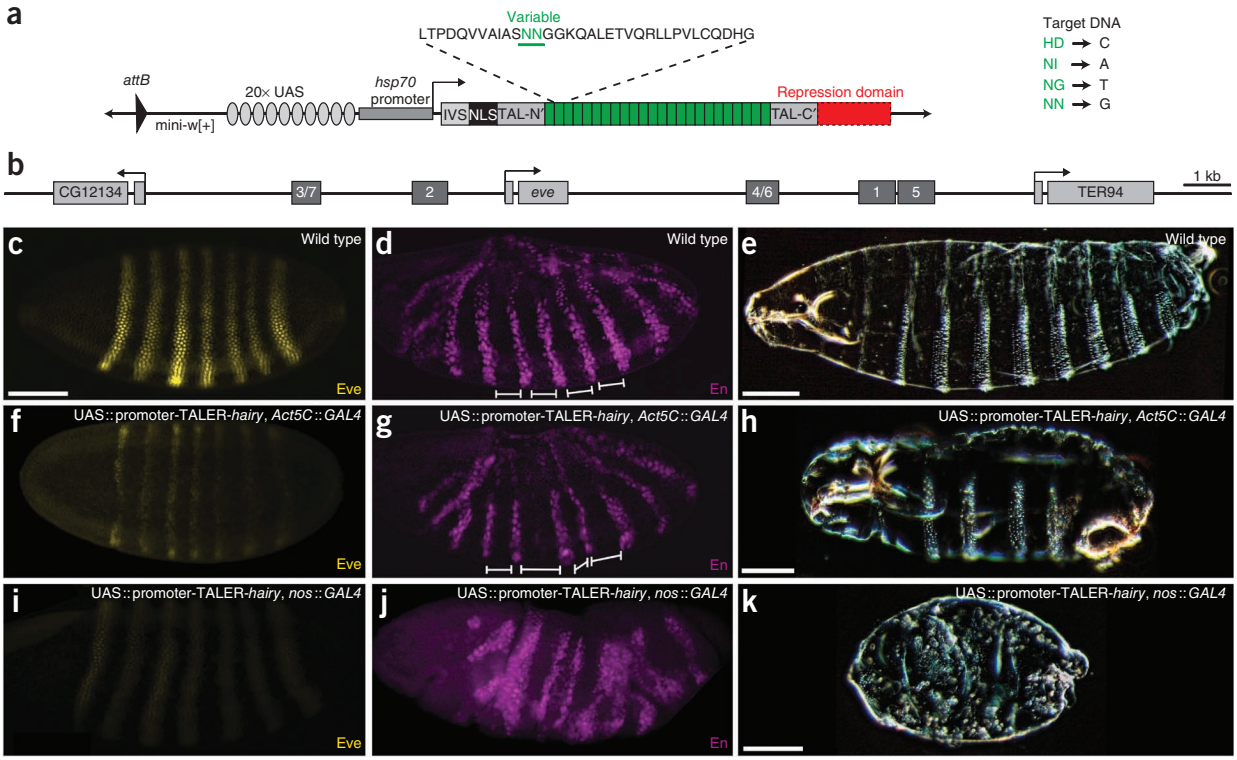

Figure 1 | TALERs targeted to the promoter can repress expression of *eve*. (**a**) Schematic of a GAL4-responsive TALER construct. UAS, upstream activating sequence; IVS, intervening sequence; NLS, nuclear localization signal. (**b**) Schematic of the *eve* locus, indicating early embryonic *cis*-regulatory stripe enhancers. (**c**–**k**) Micrographs showing stage 5 embryos stained for Eve (**c**,**f**,**i**), stage 11 embryos stained for En (**d**,**g**,**j**) and larval cuticle preps (**e**,**h**,**k**) in the indicated genotypes. Scale bars, 100 µm. Embryos in **c**,**d**,**f**,**g**,**i**,**j** are matched in scale.

TALER-mediated repression of the *eve* promoter

To determine the efficiency of TALERs in the embryo, we drove ubiquitous, zygotic expression of a TALER-Hairy fusion protein targeted near the *eve* promoter. This TALER-Hairy reduced expression of all *eve* stripes and resulted in abnormal expression of *engrailed* (*en*), a target of Eve[23] (**Fig. 1f**,**g**). Larval cuticles of these embryos exhibited fused segments (**Fig. 1h**). 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 with a maternally expressed driver, *nanos-GAL4* (*nos*::*GAL4*)[26]. In these

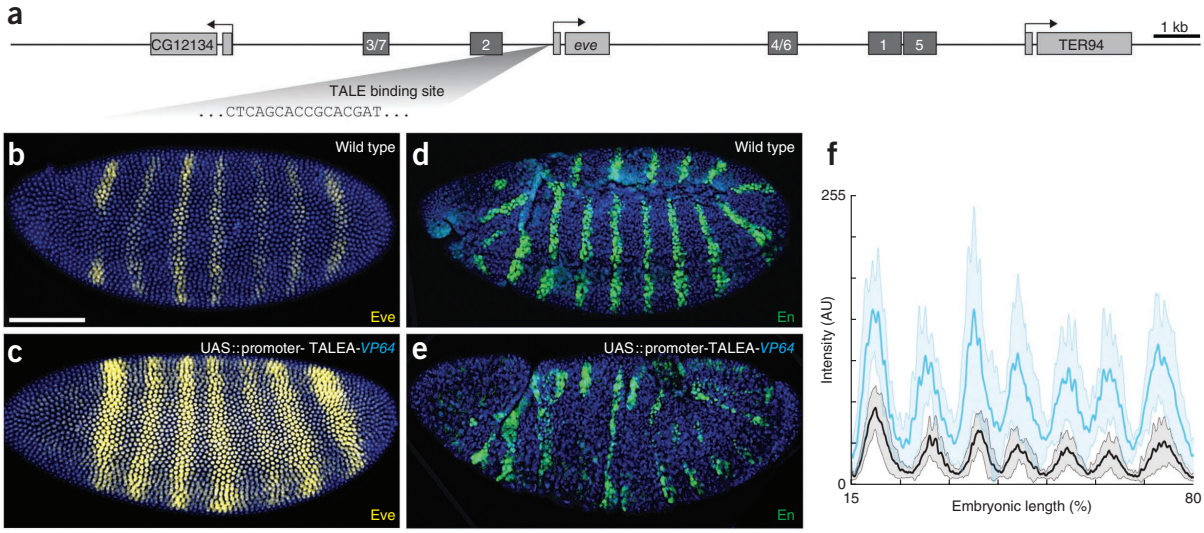

Figure 2 | TALE targeted activation of the *eve* promoter. (**a**) Schematic of the *eve* locus, indicating early embryonic *cis*-regulatory stripe enhancers and the TALE binding site. (**b**,**c**) Stage 5 wild-type embryo (**b**) and embryo carrying upstream activating sequence (UAS)::promoter-TALEA-*VP64*, *nos*::*GAL4* (**c**) stained for Eve. (**d**,**e**) Stage 12 wild-type embryo (**d**) and UAS::promoter-TALEA-*VP64*, *nos*::*GAL4* embryo (**e**) stained for En. (**f**) Profiles of average expression of Eve in stage 5 embryos (n = 10 for each genotype). The black line denotes wild-type embryos, and the turquoise line denotes the promoter-TALEA-*VP64* embryos. Shaded bounding areas indicate ±1 s.d. Signal intensity is reported in arbitrary units (AU). Scale bar, 100 µm. Embryos in **b**–**e** are matched in scale.

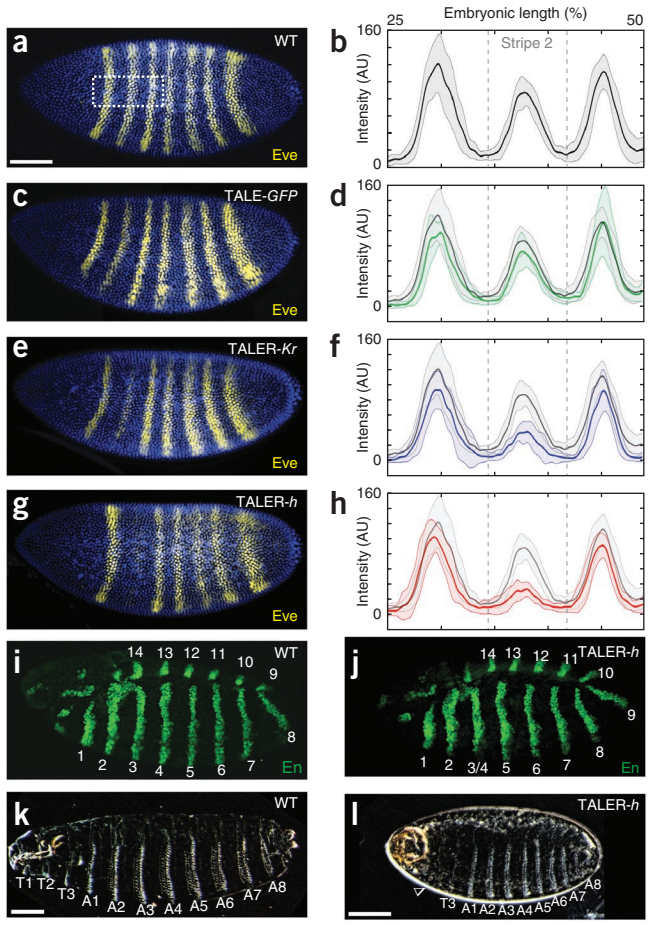

Figure 3 | TALER targeted repression of the *eve* stripe 2 enhancer. (**a**–**h**) Micrographs showing stage 5 embryos stained for Eve juxtaposed with expression profiles. Shown are a wild-type (WT) embryo (**a**) and embryos carrying upstream activating sequence (UAS)::enhancer-TALER-*GFP*, *nos*::*GAL4* (**c**), UAS::enhancer-TALER-*Krüppel* (*Kr*), *nos*::*GAL4* (**e**) or UAS::enhancer-TALER-*hairy* (*h*), *nos*::*GAL4* (**g**). The plots (**b**,**d**,**f**,**h**) show profiles of average expression in the region outlined in **a** for the corresponding genotype (n = 10 for each genotype). In all plots, the black line denotes WT embryos, and the green (**d**), blue (**f**) and red (**h**) lines denote enhancer-TALER-*GFP*, enhancer-TALER-*Krüppel* and enhancer-TALER-*hairy*, respectively. Shaded bounding areas indicate ±1 s.d. AU, arbitrary units of fluorescence intensity. (**i**–**l**) En protein staining in stage 12 embryos (**i**,**j**) and cuticle preps of first instar larvae (**k**,**l**) of the indicated genotypes. The empty arrowhead points to fused thoracic segments in the UAS::enhancer-TALER-*hairy*, *nos*::*GAL4* larva. Scale bars, 100 µm. Embryos in **a**,**c**,**e**,**g**,**i**,**j** are matched in scale.

embryos, *eve* expression was almost undetectable (**Supplementary Fig. 2**), *en* expression was severely disrupted, and outward signs of segmentation in the larval cuticle were lost (**Fig. 1i**–**k**). These results are consistent with the effects of *eve* hypomorphic alleles[27]. We also drove this TALER-Hairy using neurogenic *GAL4* drivers, and, in all cases, we observed decreased Eve expression 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*[28]. 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 next examined 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. These embryos exhibited stronger and broader patterns of expression of all seven stripes of Eve than did wild-type embryos (**Fig. 2**). Although we observed low levels of Eve expression between the canonical stripes, we still observed a clear seven-stripe pattern of expression. Expression of En was disrupted in these embryos (**Fig. 2e**), as expected[29].

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*[19]. 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 in the *eve* stripe 2 enhancer[30,31]. Both TALERs repressed *eve* stripe 2 expression specifically, and the TALER-Hairy generated stronger repression than did the TALER-Krüppel (**Fig. 3a**–**h**). We observed no notable changes in the expression of other *eve* stripes (**Fig. 3a**–**h**), even though the enhancer for stripes 3 and 7 ('3/7 enhancer') is located only 1.6 kb upstream from the targeted binding site (**Fig. 1b**). These embryos lost a single stripe of *en* expression (**Fig. 3i**,**j**), which is consistent with the En phenotype produced by a deletion of *eve* stripe 2 (ref. 31). Furthermore, these embryos failed to hatch, and larval cuticles exhibited an altered gnathal segment (**Fig. 3k**,**l**), as expected[27]. As a control, ubiquitous expression of a TALE-GFP fusion protein targeted to the same 16-bp sequence in *eve* stripe 2 did not alter *eve* expression (**Fig. 3c**,**d**). 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.

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* were expressed in fewer cell rows, a result consistent with previous observations that *eve* enhancers are sensitive to repressor concentrations[32]. A TALER-Hairy targeted to the minimal autoregulatory sequence, located approximately 5 kb upstream of the *eve* promoter, caused a strong reduction in expression of all *eve* stripes after embryonic stage 5, as expected[33] (**Supplementary Fig. 6**). We found that a TALER-Hairy construct targeting the stripe 4/6 enhancer caused a slight reduction in *eve* stripe 5 expression (**Fig. 4c**). However, TALERs targeting two different binding sites in the 4/6 enhancer produced similar patterns of repression of stripes 4 and 6 (**Supplementary Fig. 5**), whereas only one of these TALERs reduced expression of

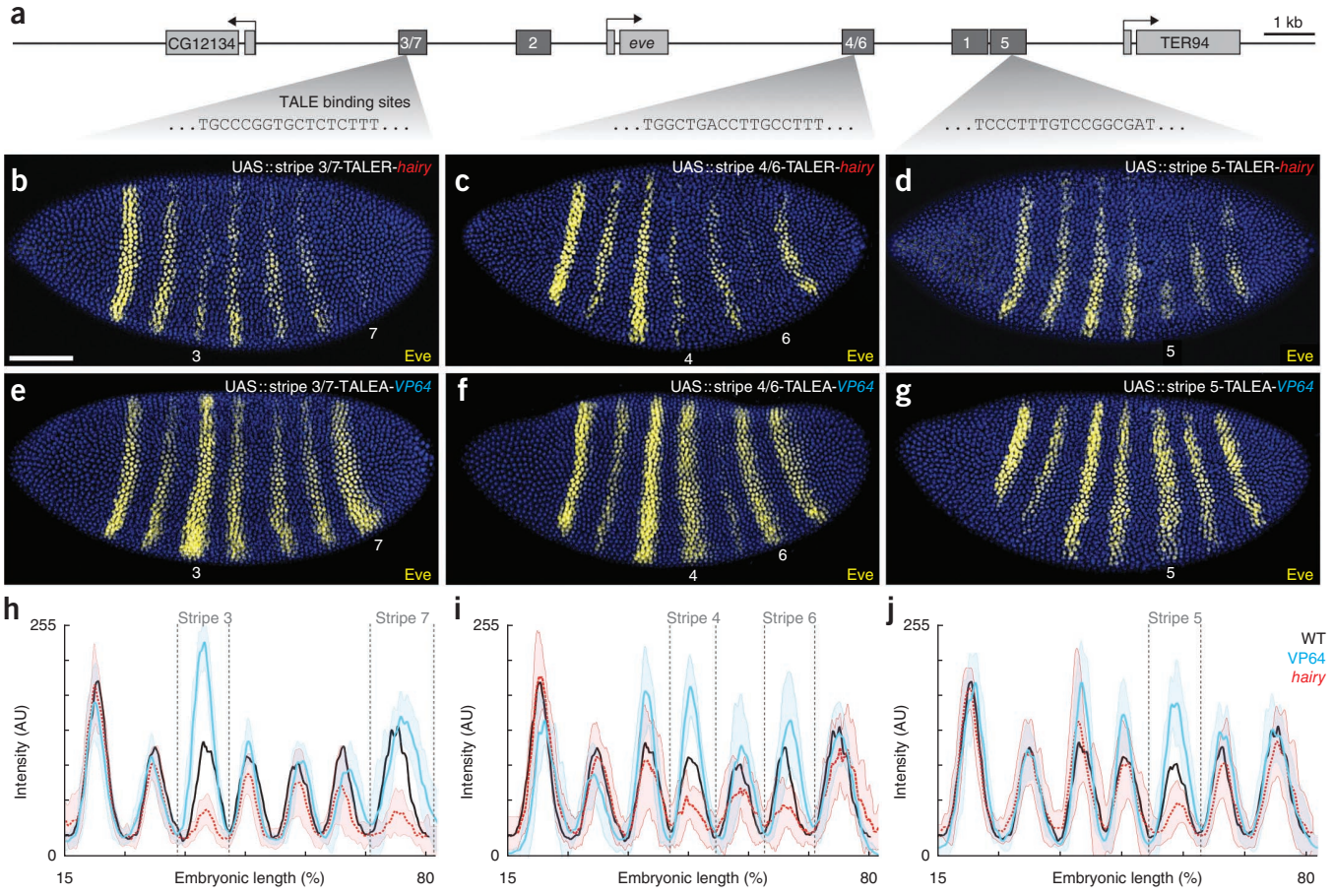

Figure 4 | TALE targeted repression and activation of *eve* stripe enhancers. (**a**) Schematic of the *eve* locus, indicating early embryonic *cis*-regulatory stripe enhancers and TALE binding sites. (**b**–**g**) Stage 5 embryos stained for Eve and carrying *nos*::*GAL4* and either upstream activating sequence (UAS)::stripe 3/7-TALER-*hairy* (**b**), UAS:: stripe 4/6-TALER-*hairy* (**c**), UAS::stripe 5-TALER-*hairy* (**d**), UAS::stripe 3/7-TALEA-*VP64* (**e**), UAS::stripe 4/6-TALEA-*VP64* (**f**) or UAS::stripe 5-TALEA-*VP64* (**g**). (**h**–**j**) Profiles of average expression 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**) or UAS::stripe 5-TALE, *nos*::*GAL4* (**j**) (n = 10 for each genotype). In all plots, the black line denotes wild-type embryos, and the turquoise and dotted red lines denote enhancer-TALEA-*VP64* and enhancer-TALER-*hairy*, respectively. Shaded bounding areas indicate ±1 s.d. AU, arbitrary units. Scale bar, 100 µm. Embryos in **b**–**g** are matched in scale.

stripe 5. Although 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, a finding that suggests that, in a native genomic context, Hairy may function at a more limited range—or with greater specificity—than has been suggested previously[25].

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[32]. Although the quantitative level of activators is clearly important for determining levels of gene expression[34], it is thought that most activators are unable to overcome the limiting effects of repressors[32]. 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 active already. 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 expression specifically in the stripe driven by the native enhancer (**Fig. 4e**–**j**). In several cases, the targeted *eve* stripe was expressed in more cell rows for transgenic embryos than for 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 increased mainly stripe 3 expression, and the TALEA targeting the 4/6 enhancer increased mainly stripe 4 expression (**Fig. 4h** and **4j**). There are several possible explanations for these observations. First, although 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**). Notably,

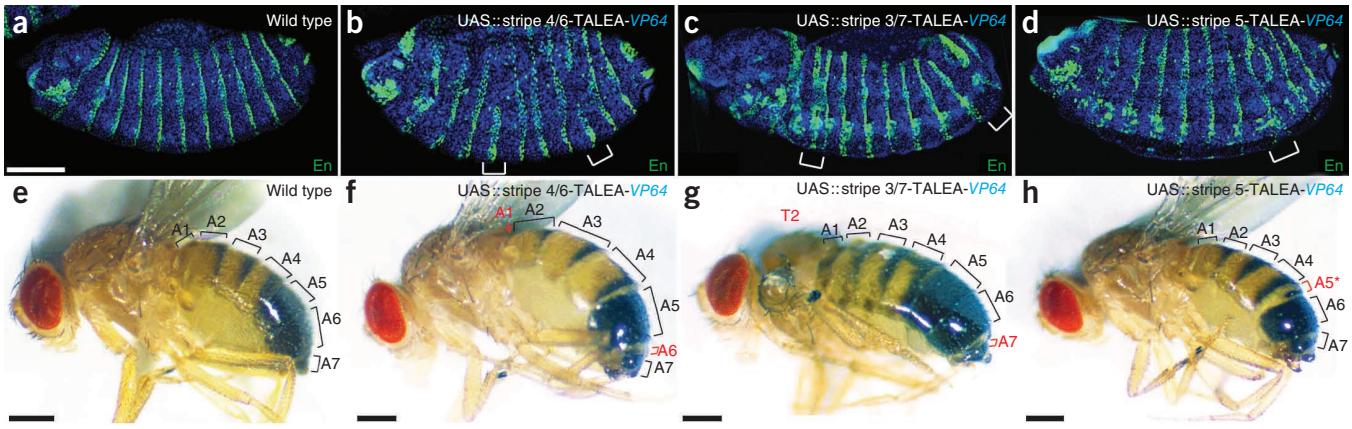

Figure 5 | Phenotypes resulting from enhancer-TALEA-*VP64* activation of *eve* enhancers. (**a**–**d**) Stage 15 wild-type embryo (**a**) and embryos carrying upstream activating sequence (UAS)::stripe 4/6-TALEA-*VP64*, *nos*::*GAL4* (**b**), UAS::stripe 3/7-TALEA-*VP64*, *nos*::*GAL4* (**c**) or UAS::stripe 5-TALEA-*VP64*, *nos*::*GAL4* (**d**) stained for En. Altered and fused parasegments are indicated with white brackets. (**e**–**h**) Adult wild-type fly (**e**) and adults carrying UAS::stripe 4/6-TALEA-*VP64*, *nos*::*GAL4* (**f**), UAS::stripe 3/7-TALEA-*VP64*, *nos*::*GAL4* (**g**) or UAS::stripe 5-TALEA-*VP64*, *nos*::*GAL4* (**h**). Adult abdominal (A) segments are labeled, with fused and altered abdominal and thoracic (T) segments in red. Scale bars, 100 μm (**a**) and 400 μm (**e**–**h**). Embryos in **a**–**d** are matched in scale.

adult flies developed from embryos treated with each of the three TALEAs: TALEA stripe 4/6 adults displayed reduced abdominal segments 1 and 6 (**Fig. 5e**,**f**); TALEA stripe 3/7 adults displayed fusion of the T2 and T3 segments, including loss of a pair of legs, and reduced abdominal segment 7 (**Fig. 5g**); and TALEA stripe 5 adults exhibited a reduced abdominal segment 5 (**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[35] (**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 the binding observed for metazoan transcription factors, which seem to have evolved relatively low specificity protein-DNA interactions to enable cooperative and synergistic binding[36]. The relatively local effects of the enhancer-TALER-*hairy* constructs that we observed are inconsistent with previous reports of long-range repression by *hairy*[37]. We suggest two hypotheses to explain this discrepancy. First, enhancers may bind proteins—either directly through 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* stripe 2 leads to lower transcriptional robustness[31].

Perhaps the most interesting finding is that none of the ubiquitously expressed TALEAs targeted to single enhancers disrupted all seven stripes of *eve* expression or drove expression in other ectopic locations. Even the TALEA targeted to the promoter drove increased expression in mainly the seven-stripe region. There are at least two possible explanations for these results. First, TALEAs may bind to their respective targets in all embryonic cells, but their activating signals may be over-ridden by repressive cues. Alternatively, the TALEA binding sites may be inaccessible to TALEA binding in cells in which 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[38,39].

Our results strongly support a model for combinatorial activation of independent, modular *Drosophila eve* enhancers[4,24,25]. The precise effects of the TALEAs supports the view that repression acts in a dominant fashion on transcriptional activators[32,40]. Because TALERs and TALEAs provide experimental access specifically to active enhancers, they may allow functional dissection of nonmodular enhancer architectures that have confounded reporter-gene assays.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. TALE plasmids are available at Addgene: 46145 (pJC-TALE-*hairy*), 46146 (pJC-TALE-*Kr*), 46147 (pJC-TALE-*VP64*) and 46148 (pJC-TALE-*GFP*).

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.C. conceived of, designed and executed the experiments and analyzed the data, with mentorship from D.L.S. J.C. and D.L.S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE 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] through mutation of all Esp3I sites. TALE–C terminus fusion proteins were synthesized by GenScript and subcloned into JFRC-7 at the XhoI/XbaI sites with removal of the mCD8 and GFP domains. The following domains were added in separate constructs: GFP[41]; Kr repression domain, amino acids 402–502; Hairy repression domain 255–337; and VP64 activation domain[41].

Construction of TALEs. TALE target sites were identified using the TAL Effector-Nucleotide Targeter, TALE-NT[18]. TALEs were subsequently assembled using the Golden Gate method[18].

Fly strains and crosses. *D. 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: *Actin 5C–GAL4*; NGT40 (Bloomington stock 4442; ref. 26); 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. Embryos were 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, which was followed by detection of primary antibodies using secondary antibodies labeled with Alexa Fluor dyes (1:500, Invitrogen). Cuticle preps were performed using standard protocols.

Microscopy. Confocal images were obtained on a Leica DM5500 Q Microscope with an ACS APO 20×/0.60 IMM CORR lens and Leica Microsystems LAS AP software. Sum projections of confocal stacks were assembled, embryos were scaled to match sizes, background was subtracted using a 50-pixel rolling-ball radius and plot profiles of fluorescence intensity were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/). Data from the plot profiles were further analyzed in Matlab.

41. Pfeiffer, B.D. *et al.* Refinement of tools for targeted gene expression in *Drosophila*. *Genetics* **186**, 735–755 (2010).