Please cite this article in press as: Li and Arnosti, Long- and Short-Range Transcriptional Repressors Induce Distinct Chromatin States on Repressed Genes, Current Biology (2011), doi:10.1016/j.cub.2011.01.054

Current Biology *21*, 1–7, March 8, 2011 ©2011 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2011.01.054

Report

Long- and Short-Range Transcriptional Repressors Induce Distinct Chromatin States on Repressed Genes

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Summary

Transcriptional repression is essential for establishing precise patterns of gene expression during development [1]. Repressors governing early *Drosophila* segmentation can be classified as short- or long-range factors based on their ranges of action, acting either locally to quench adjacent activators or broadly to silence an entire locus [2]. Paradoxically, these repressors recruit common corepressors, Groucho and CtBP, despite their different ranges of repression [3–7]. To reveal the mechanisms underlying these two distinct modes of repression, we performed chromatin analysis using the prototypical long-range repressor Hairy and the short-range repressor Knirps. Chromatin immunoprecipitation and micrococcal nuclease mapping studies reveal that Knirps causes local changes of histone density and acetylation, and the inhibition of activator recruitment, without affecting the recruitment of basal transcriptional machinery. In contrast, Hairy induces widespread histone deacetylation and inhibits the recruitment of basal machinery without inducing chromatin compaction. Our study provides detailed mechanistic insight into short- and long-range repression on selected endogenous target genes and suggests that the transcriptional corepressors can be differentially deployed to mediate chromatin changes in a context-dependent manner.

Results and Discussion

Local and Global Repression

To directly compare functional aspects of Hairy- and Knirps-mediated repression in the *Drosophila* embryo, we studied these proteins' interactions with two segmentally expressed pair-rule genes. Hairy directly represses *fushi tarazu* (*ftz*), a secondary pair-rule gene expressed in the blastoderm embryo in a seven-stripe pattern [8]. *ftz* is regulated by both regionally acting gap genes and the segmentally expressed *hairy* pair-rule gene [9]. Chromatin immunoprecipitation (ChIP) experiments have revealed dense clusters of peaks around the *ftz* gene for key transcription factors active in the blastoderm embryo, including Caudal, Hunchback, Knirps, Giant, Huckebein, Krüppel, and Tailless. These transcription factors bind to the promoter-proximal Zebra element, the stripe 1+5 enhancer located 3′ of *ftz*, and a presumptive 5′ regulatory region located between −3 kbp and −8 kbp [10–12] (Figure 1A). Hairy has been found to bind in vivo to all of these regions. This repressor is expressed in a striped pattern in the blastoderm embryo; therefore, the *ftz* gene is active in some nuclei and repressed in others. In order to obtain a homogeneous population of nuclei for chromatin studies, we overexpressed Hairy protein in embryos using a heat-shock driver, which results in complete repression of *ftz* (Figure 1A). This repression requires the recruitment of the Groucho corepressor, because a mutant version of Hairy that does not bind to Groucho fails to repress *ftz* (Figures 1E and 1F).

Interestingly, a titration of heat-shock induction resulted in a nonuniform, progressive loss of specific *ftz* stripes, with stripe 4 being the most sensitive and stripe 1+5 the least (Figure 1C; differential repression is quantified in Table S1 available online). This result points to the intriguing possibility that Hairy can act locally on specific enhancers, at least very transiently, although the end result of Hairy repression is complete silencing of all enhancer elements. The asynchronous repression of the *ftz* locus also suggests that Hairy-mediated long-range repression does not act solely by direct targeting the basal promoter, as suggested by a previous model for this class of repressor, because this mechanism should cause uniform inhibition of stripe elements [13].

Similar to *ftz*, the pair-rule gene *even skipped* (*eve*) is also expressed in a seven-stripe pattern and is regulated by multiple modular enhancers (Figure 1B). *eve* is a well-characterized target of the short-range repressor Knirps, which sets posterior boundaries of *eve* stripe 3 and 4 and anterior borders of *eve* stripe 6 and 7 [14, 15]. After substantial overexpression of Knirps (20 min heat-shock induction), the repressor is able to repress all of the *eve* stripe enhancers except for the stripe 5 enhancer (Figure 1B). When the induction is titrated, Knirps represses individual enhancers in a stepwise manner, with the most sensitive enhancers downregulated earliest, at a low dose of Knirps (differential repression has been quantified in Table 1 of [16]). Together, these experiments indicate that Hairy can initially act locally but ultimately acts in a globally dominant fashion, whereas Knirps acts in a restricted manner (Figure 1D).

Hairy and Knirps Differentially Affect Chromatin Structure

To compare the effects of repression by Hairy and Knirps, we studied chromatin changes associated with repression of *ftz* and *eve* via ChIP. We observed no significant change of histone H3 occupancy at regions sampled throughout the *ftz* locus after Hairy overexpression (Figure 2A) (although some regions showed modest differences, none had $p < 0.1$; statistical significances are given in Table S2.1). In contrast, Knirps repression of *eve* resulted in significantly increased histone H3 density, particularly in two of the three regions corresponding to the Knirps-sensitive enhancers, namely stripe 4+6 and stripe 2 (Figure 2B; Table S2.1). Little change was noted in the promoter region, transcribed region, or the stripe 1 and 5 enhancers, which are not readily repressed by Knirps. An apparent increase in histone H3 density on the repressed stripe 3+7 enhancer, although of low statistical significance, correlates with other alterations common to repressed enhancers, noted below (Figure 2B; Table S2.1).

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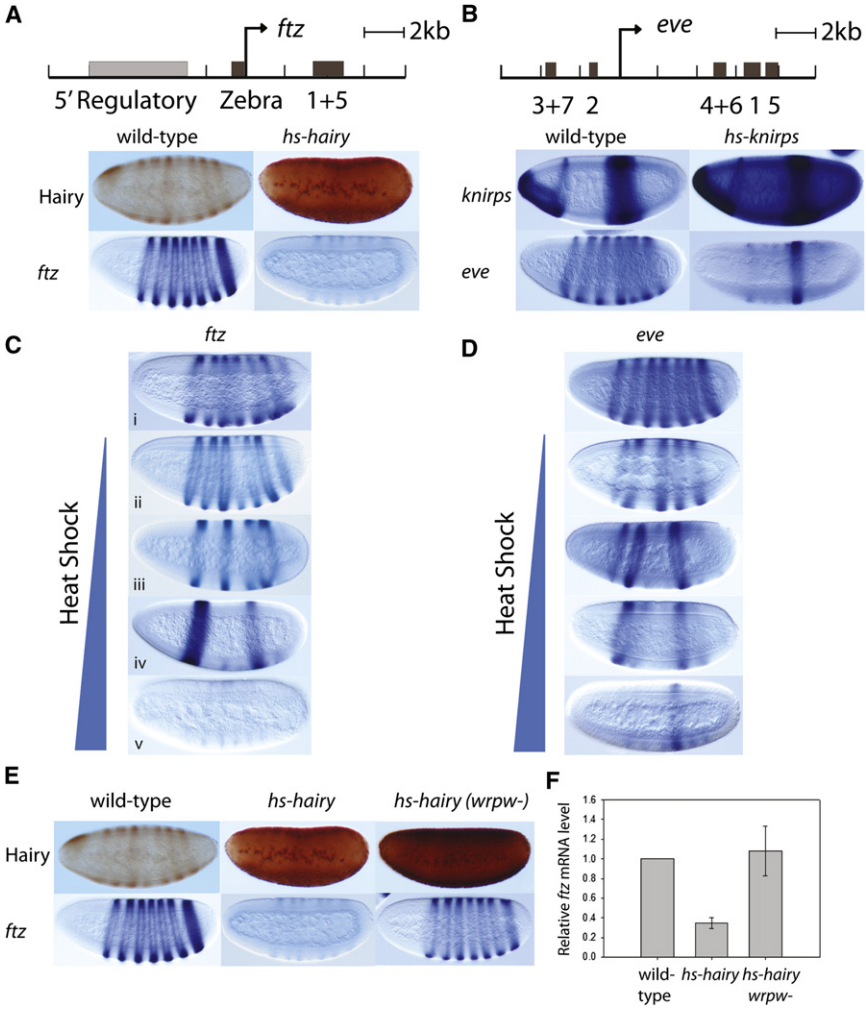

Figure 1. Repression of *ftz* by Hairy and *eve* by Knirps

(A) Overexpression of the Hairy long-range repressor abolishes *ftz* expression. Hairy protein (upper images) and *ftz* mRNA (lower images) expression pattern in wild-type and *hs-hairy* transgenic embryos after 20 min heat shock are shown. In schematic at top, characterized enhancers are shown as black blocks; the gray block denotes the presumptive 5′ regulatory region of *ftz*.

(B) Substantial induction of Knirps represses all *eve* enhancers except the stripe 5 enhancer. *knirps* (upper images) and *eve* (lower images) mRNA expression pattern in wild-type and *hs-knirps* transgenic embryos after 20 min heat shock are shown.

(C) Overexpression of the long-range Hairy repressor by titrated heat shock results in progressive repression of *ftz* stripes as shown by in situ hybridization. The order of repression is, from top to bottom, stripe 4, stripe 2+7, stripe 3+6, and stripe 1+5. Detailed results of the heat-shock titration experiment are shown in Table S1.

(D) Heat-shock titration of Knirps represses *eve* in a stepwise manner, with stripe 3+7 being the most sensitive and stripe 1 the least sensitive (from top to bottom) of the repressed stripes. Detailed results of the heat-shock titration experiments are described in [16].

(E) A mutant form of Hairy that is unable to bind Groucho fails to repress *ftz*. Hairy protein (upper images) and *ftz* mRNA (lower images) expression patterns in wild-type nontransgenic embryos, *hs-hairy*, and *hs-hairy (wrpw−)* (Groucho binding mutant) transgenic embryos after 20 min heat shock are shown.

(F) *ftz* mRNA levels in wild-type, *hs-hairy*, and *hs-hairy (wrpw−)* embryos quantified by real-time PCR, with wild-type levels normalized to 1. *ftz* mRNA levels are lower only in embryos containing the *hs-hairy* transgene. Heat shock alone, in the absence of the *hs-hairy* or *hs-knirps* transgenes, had no effect on *ftz* and *eve*. Error bars represent standard error.

To provide a more detailed picture of chromatin structure, we adapted a micrococcal nuclease (MNase) mapping protocol used in yeast and cultured cells for *Drosophila* embryos [17–19]. MNase mapping showed that Hairy repression had little effect on chromatin accessibility throughout the *ftz* locus (Figure 2C; Table S2.2), whereas Knirps induced a significant increase in MNase insensitivity specifically at the *eve* stripe 3+7, 2, and 4+6 enhancers and a minor increase in stripe 1 protection (Figure 2D; Table S2.3). The promoter and the *eve* stripe 5 enhancer were little changed, mirroring the patterns noted for overall histone H3 occupancy. The changes noted for the *eve* locus appear to be specific, because Knirps did not induce any change of a nontargeted intergenic site on the third chromosome. Hairy also had no effect at this locus (Figure 2C).

The similar results from overall histone H3 density and MNase mapping suggest that Hairy-mediated long-range repression does not involve a general compaction of chromatin on the *ftz* locus. In contrast, repression by Knirps is associated with an increase in the histone density of targeted enhancer regions, which may result either from Knirps recruitment of factors that mediate chromatin condensation or the blocking of proteins responsible for loosening of chromatin. Recruitment of Groucho by other repressor proteins is also associated with distinct effects: Runt-dependent repression of *slp1* does not involve changes in H3 density, but Brinker repression of the *vgQ* enhancer does [20, 21]. The distance dependence of these repressors has not been established, but in light of our results, it is apparent that the Groucho corepressor can be involved in distinct effects depending on the context of recruitment [22].

Hairy Mediates Widespread Histone Deacetylation, Whereas Knirps Induces Local Histone Deacetylation

Histone acetylation is dynamically regulated on transcribed genes in eukaryotes, with histone acetylation generally correlated with active loci [23]. The histone deacetylase Rpd3 is a component of both Hairy and Knirps corepressor complexes; therefore, we assayed histone acetylation levels across the *eve* and *ftz* genes before and after repression [24, 25]. Hairy repression resulted in widespread histone H4 deacetylation throughout the *ftz* locus (Figure 3A; Table S2.1). The ectopically expressed Hairy protein itself was not observed to spread but remained restricted to regions of the gene previously observed to bind endogenous Hairy (Figure S2). Using anti-H3-acetylation antibodies, similar widespread H3 deacetylation was also noted (data not shown). This distributed effect on the *ftz* locus correlates with prior observations that Hairy-mediated long-range repression might

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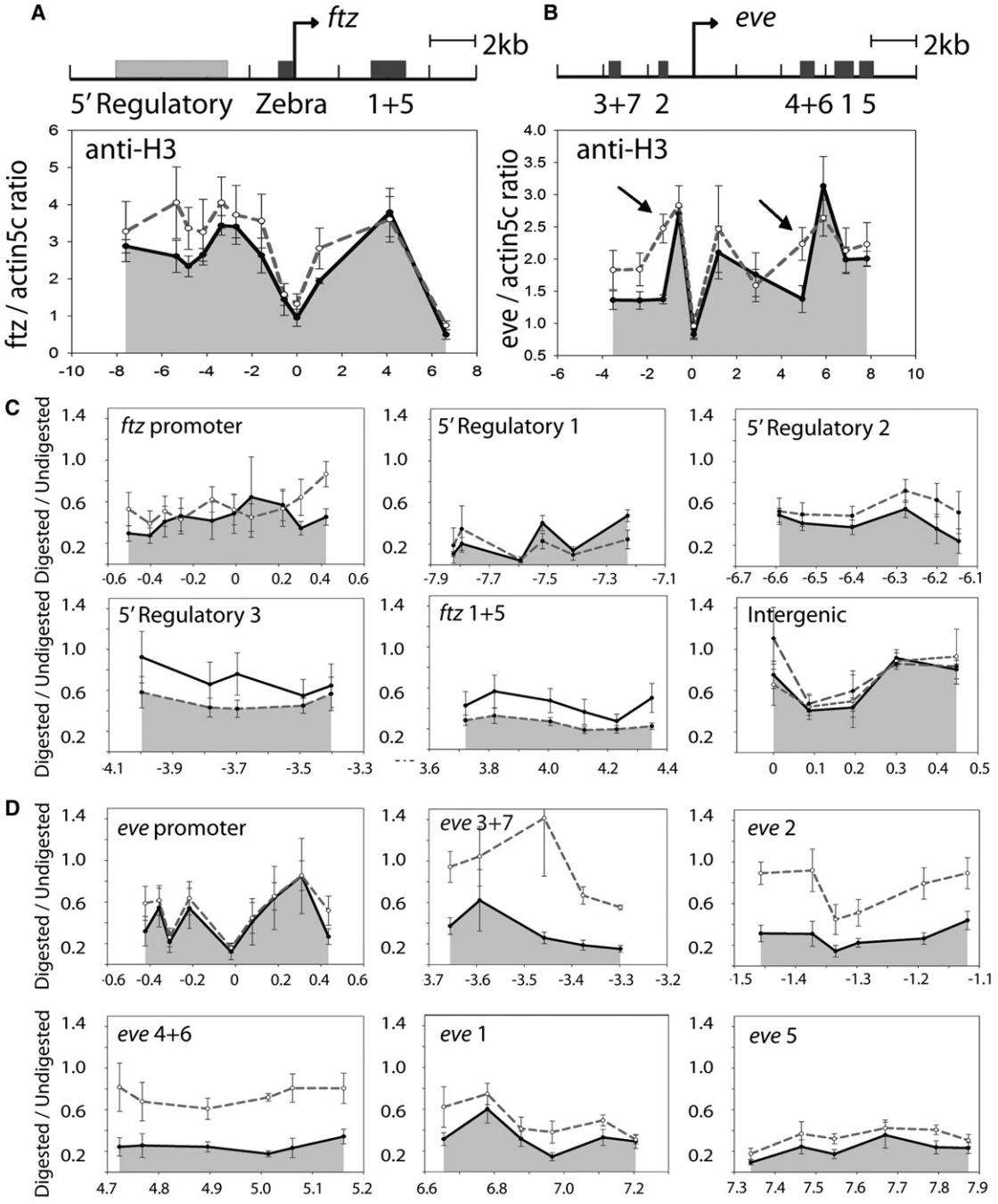

Figure 2. Hairy- and Knirps-Mediated Transcriptional Repression Result in Differential Changes in Histone H3 Occupancy and Micrococcal Nuclease Sensitivity

(A and B) Histone H3 occupancy was measured by chromatin immunoprecipitation (ChIP) in regions of *ftz* and *eve* before (solid line) and after (dashed line) repression. Significant changes were observed specifically on *eve* stripe 2 and 4+6 enhancers repressed by Knirps (arrows), but H3 occupancy was not significantly altered on *ftz* after repression by Hairy. y axis shows the relative immunoprecipitation signals normalized to the *actin5C* promoter region, which was not affected by Hairy or Knirps repression.

(C and D) Micrococcal nuclease (MNase) sensitivity of the *ftz* and *eve* loci. Hairy-induced repression is not associated with significant change in the overall MNase sensitivity pattern in any of the regions tested in the *ftz* locus (solid line before repression, dashed line after). Knirps repression is associated with increased resistance to MNase digestion specifically at the *eve* 3+7, 2, and 4+6 enhancers. Little or no change is observed at the promoter, stripe 1 enhancer, and stripe 5 enhancer. The specificity of MNase digestion was also shown by the digestion pattern of a 450 bp intergenic region on the third chromosome as shown in (C) for embryos with no repressor overexpression (solid line), Hairy overexpression (long dashed line), and Knirps overexpression (short dashed line). y axis shows the MNase-digested/undigested ratios.

Results represent at least three biological replicates; error bars show standard errors. Areas under the lower plots are shadowed for clarity of presentation. For this and later figures, the statistical significance of the differences between each pair of points is shown in Tables S2.1–S2.3; for histone H3 occupancy, $p < 0.05$ for *eve* stripes 2 and 4+6; for *ftz*, no points reached this level of significance.

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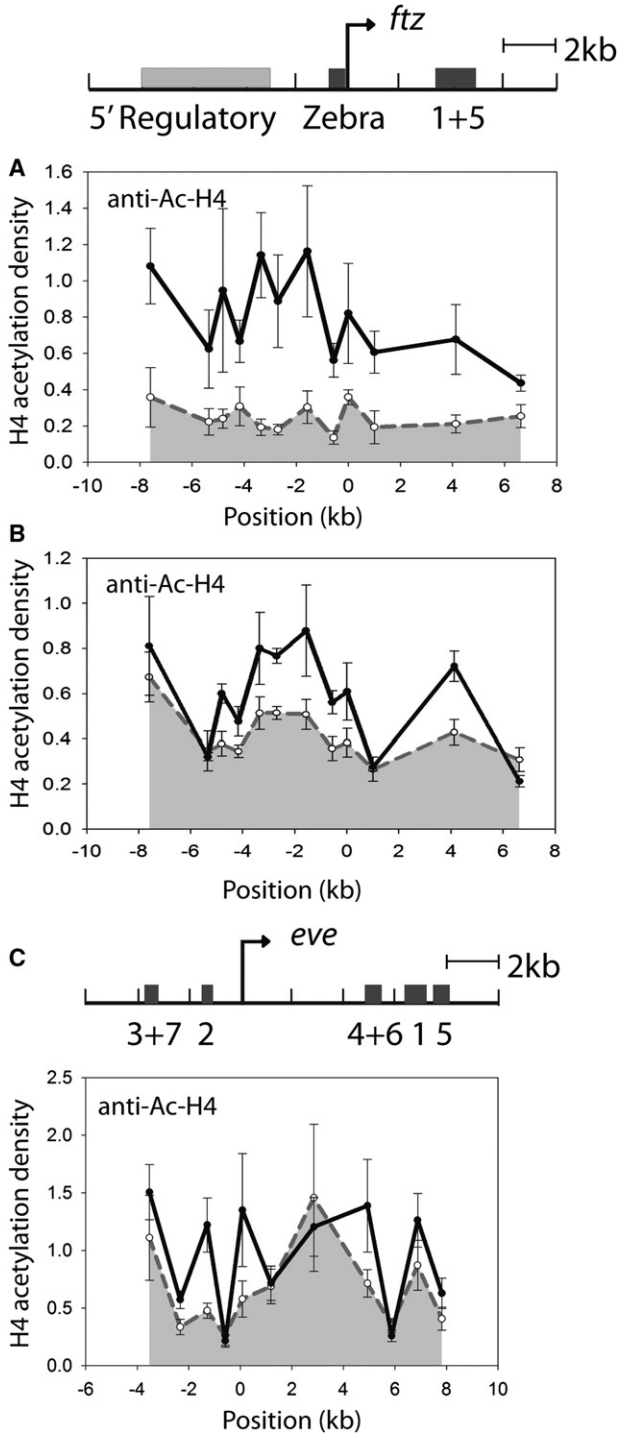

Figure 3. Hairy induces Global Changes Whereas Knirps Induces Local Changes in Histone Acetylation Levels

(A) H4K5, -8, -12, and -16 acetylation was assayed by ChIP on the *ftz* locus before (solid line) and after (dashed line) repression by Hairy. Significant reduction in H4 acetylation was observed at all positions tested around *ftz*.
(B) After a brief induction of Hairy, H4 deacetylation is found to be localized to the stripe 1+5 enhancer and the 5′ regulatory region of *ftz*.
(C) Reduction in H4 acetylation on *eve* is especially pronounced at repressed enhancers. y axis shows the H4 acetylation density, which is obtained by normalizing immunoprecipitation signals first to the H4 acetylation levels at the *actin5C* promoter region and then to the relative H3 levels. Results represent at least three biological replicates; error bars show standard errors.

involve a Groucho-mediated "spreading" mechanism [26]. By this means, Rpd3 may be delivered to extensive areas of a gene. To test whether a spreading of histone deacetylation might correlate with the successive inhibition of *ftz* enhancers that we noted in Figure 1, we investigated histone acetylation levels across *ftz* after a brief 5 min heat shock followed by immediate fixing, before the entire complement of enhancers can be repressed. In this setting, deacetylation was mostly concentrated around the stripe 1+5 enhancer and the immediate 5′ regulatory region, areas that show Hairy occupancy in vivo [12]. More distal 5′ regulatory regions and the transcription unit itself showed little initial change, consistent with a spreading action of this repressor during the more extensive repression period (Figure 3B; Table S2.1).

A different picture emerged from studies of Knirps acting on *eve*. Here, repression led to selective decreases in H3 and H4 acetylation levels, concentrated over the *eve* stripe 4+6 and stripe 2 enhancers, with lesser decreases noted at stripe 3+7 and stripe 1 enhancers (Figure 3C; Table S2.1). A local change in acetylation was also noted near the transcriptional initiation site, but not immediately 5′ and 3′ of this area. The reductions in histone acetylation levels seen on both *eve* and *ftz* are consistent with Hairy and Knirps recruiting deacetylases to their target genes. However, it is striking that the broad deacetylation mediated by Hairy on *ftz* is not associated with dramatic changes in histone density or resistance to nuclease accessibility, whereas increased histone density and resistance to nuclease digestion are associated with Knirps repression on *eve*. It is possible that in addition to inducing deacetylation, Knirps triggers additional histone modifications or interacts with nucleosome-remodeling complexes to further alter chromatin at the enhancers. H3 lysine 27 methylation is one chromatin signature associated with silenced genes; however, no significant change in this modification was noted at *ftz* or *eve* upon repression (Figure S1).

Differential Effects of Hairy and Knirps on Activator Recruitment

Our previous studies indicated that Hairy can effectively repress a reporter gene without displacing the activators [26]. We sought to test whether this was the case on an endogenous gene, *ftz*, by examining occupancy by Caudal, a transcription factor that also activates *eve*. Caudal activates the posterior stripes of both *ftz* and *eve*, and we found that Caudal binds the *ftz* 5′ regulatory region and the promoter-proximal *Zebra* element, consistent with a recent global study [27–29]. Repression of the locus by Hairy did not affect the Caudal binding pattern (Figure 4A; Table S2.1), similar to the results obtained with a Hairy-regulated reporter gene [26]. In contrast, Knirps repression decreased Caudal occupancy specifically at the *eve* 3+7 and 4+6 enhancers (Figure 4B; Table S2.1), bringing overall protein occupancy down to near baseline levels. This decrease is not an effect of global decrease of Caudal occupancy, because the Caudal binding peak at the *eve* promoter was not affected. A similar decrease in Caudal occupancy was also observed on a *hunchback* enhancer after repression by Knirps (data not shown). Interestingly, Bicoid occupancy of the *eve* stripe 2 and stripe 1 enhancers was not altered by Knirps, although these enhancers were repressed (Figure 4C; Table S2.1). Clearly, loss of transcription factor occupancy is not required for short-range repression of a *cis*-regulatory element. It is possible that different transcriptional activators exhibit differential sensitivity to chromatin changes induced during repression.

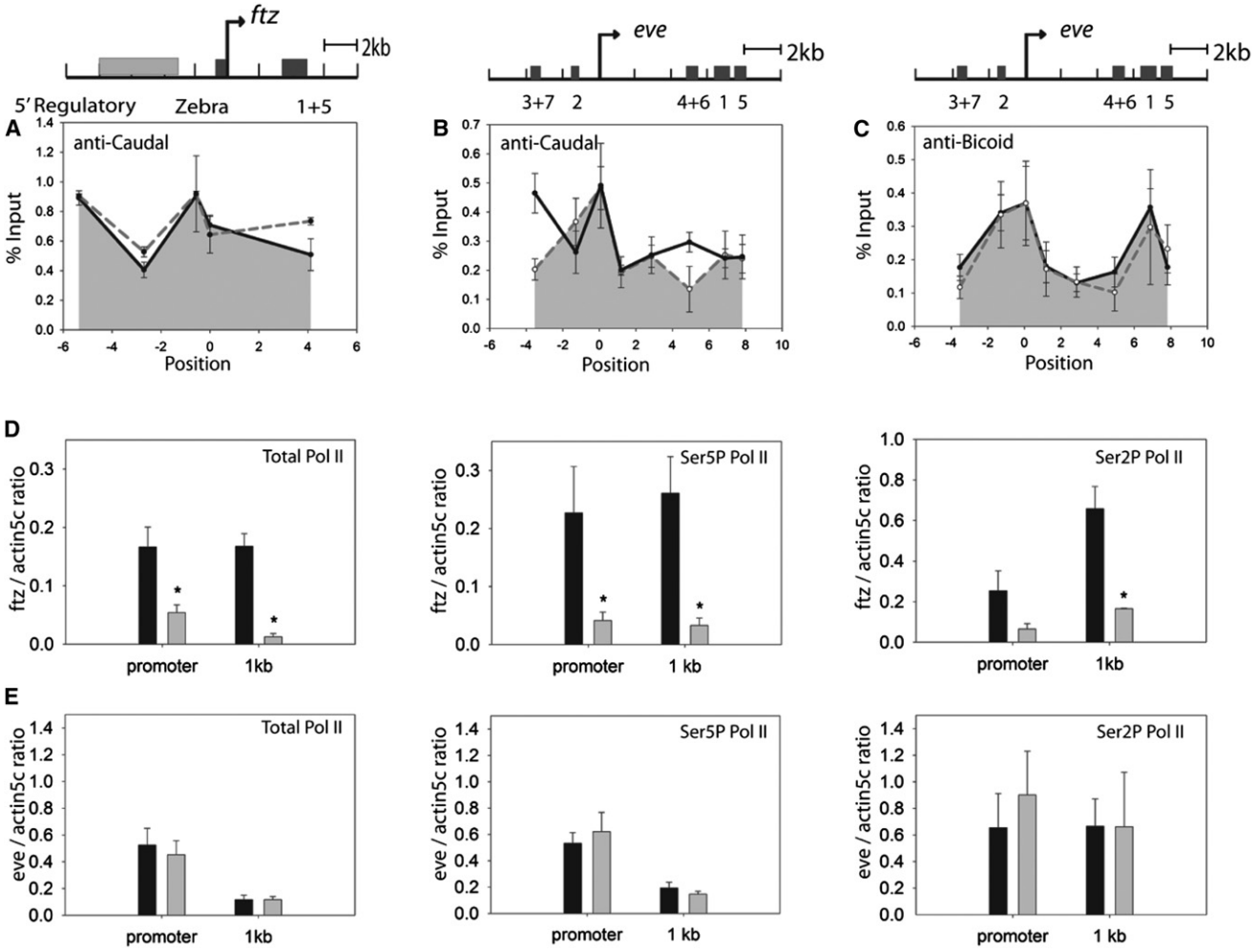

Figure 4. Activator and RNA Polymerase II Occupancy of *ftz* and *eve* before and after Repression

(A) Caudal protein occupancy of *ftz* measured by ChIP. Caudal activator levels do not decrease on the *ftz* locus in response to repression by Hairy. In (A)–(C), y axis shows immunoprecipitation signals as percentages of input. Solid lines indicate before repression; dashed lines indicate after repression. Results in (A)–(C) represent at least three biological replicates; error bars show standard errors.

(B) Caudal occupancy decreases at *eve* stripe 3+7 and 4+6 enhancers after repression by Knirps.

(C) Bicoid occupancy of *eve* is unchanged after Knirps repression. No Bicoid binding was detected on the *ftz* locus (data not shown).

(D) ChIPs were performed using antibodies against preinitiation, initiation (Ser5P), and elongation (Ser2P) RNA polymerase II (Pol II) on *ftz* before (black bars) and after (gray bars) repression. Strong decreases in all forms of Pol II were noted. *$p < 0.05$ by Student's t test. In (D) and (E), y axis shows relative immunoprecipitation signals normalized to the *actin5C* promoter region, which was not affected by Hairy or Knirps repression. Error bars show standard errors.

(E) A similar analysis of *eve* did not show significant changes in Pol II occupancy after Knirps repression.

Distinct Effects on RNA Polymerase II by Long- and Short-Range Repressors

New insights have suggested that many developmental genes, including those regulated by short-range repressors such as Snail, feature RNA polymerase paused in the promoter region even in their inactive state, suggesting postrecruitment levels of regulation [30]. We analyzed components of the core machinery before and after repression by Hairy and Knirps. Upon Hairy repression, a marked decrease of RNA polymerase II (Pol II) occupancy was observed at the *ftz* locus. The same trend was observed for the preinitiation, initiation, and elongation forms of Pol II (Figure 4D). These results suggest that Hairy directly or indirectly blocks recruitment of Pol II. Similar decreases were noted with levels of TATA box-binding protein (TBP) at the promoter (data not shown).

In contrast, induction of Knirps did not change Pol II occupancy at the *eve* transcription unit, even under condition where most enhancers were repressed (Figure 4E). (Under conditions tested here, over three-quarters of the embryos had shut down expression of all but stripe 1 and/or 5.) Similarly, TBP occupancy remained at a comparable level before and after Knirps repression (data not shown). The constant level of RNA polymerase on the *eve* transcription unit was a surprise in light of the sharp reduction in mRNA production as measured by in situ hybridization. However, there is precedence for this effect: Runt repression of *slp1* appears to act through elongation control, which causes no change of the concentration of Pol II on *slp1* [20]. Knirps may produce a similar effect by inducing a slower transit rate of Pol II on the repressed *eve* locus. Similar observations have been made at the *hsp70* gene upon depletion of elongation factors such as Spt6 or Paf1 [31, 32].

The differential distance dependence of short- and long-range repressors such as Hairy and Knirps has been observed in many contexts [13, 14, 33, 34]. However, the mechanisms by

which these proteins function have been poorly understood. With the recent demonstration that transcriptional factors considered to be short- and long-range repressors utilize shared cofactors, namely CtBP and Groucho, there has been a question of whether long-range repression is actually functionally distinct from short-range repression [6]. Our study provides evidence that the chromatin states associated with long- and short-range repressors are distinct in several ways. We do not yet know whether the effects seen on *ftz* are observed for all Hairy targets, although the similarity of changes observed on the *lacZ* reporter subject to Hairy repression suggests that they are conserved [26]. Similarly, the reproducibility of Knirps-induced changes at different *eve* enhancers indicates that this protein can effect related chromatin changes on *cis*-regulatory modules bound by different activators. Snail, another short-range repressor, also appears to mediate localized deacetylation and activator displacement; thus, this mechanism may be a common feature of this entire class of repressors ([35]; Y. Nibu, personal communication). It will be interesting to determine how general are the observations made in this study for long- and short-range repression, a question that can be approached using genome-wide methods. In any event, the highly divergent activities of Knirps and Hairy demonstrated in this study not only underscore the fact that these proteins can mediate biochemically divergent events but also raise interesting questions about how similar cofactors can participate in such distinct effects in a context-dependent manner. It is possible that the corepressors adopt distinct conformations when recruited by different repressors, or the corepressor may form distinct complexes with unique activities [22]. In addition to determining how *cis*- and *trans*-acting factors affect repression pathways, these mechanistic insights will provide important contextual information for interpretation of genome-wide transcription factor binding and chromatin modifications and will inform quantitative modeling of *cis*-regulatory elements for the aim of understanding the activity and evolution of enhancers [28, 34, 36].

Experimental Procedures

Plasmid Construction

Transgenic flies carrying inducible *hairy* genes were generated by using the pCaSpeR-hs transformation vector [37]. The genes were created by joining a EcoRI/XbaI fragment containing a Kozak sequence, initiator ATG, and a coding sequence for either wild-type or mutant (WRPW/AAAA) Hairy protein (primer sequences are listed in Table S3;) amplified from a pGEX-2T vector containing *hairy* cDNA [3]. The inducible *knirps* gene used to overexpress the protein was described in a previous study [16].

In Situ Hybridization and Antibody Staining of *Drosophila* Embryos

Embryos were fixed for in situ hybridization and stained using anti-digoxigenin-UTP-labeled RNA probe for *ftz* or *eve* as described previously [16].

Embryo Collection

Embryos used for ChIP and MNase protection experiments were 2–3 hr old, exposed to 20 min heat shock to induce maximal repression, and allowed no recovery period after heat-shock treatment. To control for possible nonspecific effects of heat shock, we similarly treated wild-type embryos without heat-shock transgenes to generate the chromatin profiles of *ftz* and *eve* in the unrepressed state (heat shock alone has no effect on the expression patterns of *eve* or *ftz*; [16] and data not shown). Embryos containing either the *hs-hairy* or *hs-knirps* transgene were used to generate the "after-repression" chromatin.

Chromatin Immunoprecipitation

Heat shocks and ChIPs were performed as described previously [26], with the exceptions that embryos were sonicated for 20 s (60% duty cycle) and cooled on ice for 30 s a total of 15 times using a Branson sonicator. After precipitation of chromatin-antibody complexes, protein A/G beads were washed twice with low-salt buffer, twice with high-salt buffer, and twice with Tris-EDTA. We used the following antibodies: mouse IgG (10 μl, Upstate), rabbit anti-H3 (1 μl, Abcam), rabbit anti-acetyl H4 (1 μl, Upstate), rabbit anti-trimethyl H3K27 (2 μl, Abcam), 8WG16 (10 μl, Covance; mouse anti-unphosphorylated Pol II CTD), H5 (10 μl, Covance; mouse anti-Ser2-phosphorylated CTD), H14 (10 μl, Covance; mouse anti-Ser5-phosphorylated CTD), mouse anti-TBP (2 μl, Abcam), rabbit anti-mouse IgM (10 μl, Abcam), rabbit anti-Bicoid serum (10 μl; gift from X. Li and M. Biggin, Genomics Division, Lawrence Berkeley National Laboratory), and rabbit anti-Caudal serum (10 μl, gift from X. Li and M. Biggin).

Micrococcal Nuclease Mapping in *Drosophila* Embryos

MNase mapping in *Drosophila* embryos was performed as described previously [17].

Quantitative PCR Analysis

The samples from ChIP and MNase mapping were analyzed via real-time PCR (Applied Biosystems 7500). Primer pairs had a melting temperature in the range of 58°C–60°C, and amplicons ranged from 50 to 150 bp. Primer sequences are listed in Table S3. For ChIP samples, a standard curve was generated by serially diluting input samples to quantify IP samples. For MNase digests, a ratio was calculated between MNase-digested and undigested samples. All values used were collected from the linear range of amplification. For the analysis of *ftz* mRNA shown in Figure 1F, mRNA was collected from 2–3 hr embryos, purified using a QIAGEN RNeasy Kit, and reversed transcribed using a High Capacity cDNA Reverse Transcription Kit from Invitrogen/Applied Biosystems. The cDNA was then analyzed by real-time PCR using the primer pair located at +1.1 kbp within the *ftz* transcription unit. Values for wild-type embryos were set to 1; results represent the average of four or more biological replicates.

Supplemental Information

Supplemental Information includes two figures and three tables and can be found with this article online at doi:10.1016/j.cub.2011.01.054.

Acknowledgments

We thank Xiaoyong Li and Mark Biggin for the Bicoid and Caudal antisera, Ze'ev Paroush for the pGEX-2T plasmid containing *hairy* cDNA, Zachary Burton and Mark Schroeder for critical reading of the manuscript, Sandhya Payankaulam for valuable advice on Knirps-related experiments and the manuscript, and other Arnosti laboratory members for useful discussions. This work is supported by National Institutes of Health grant GM56976 to D.N.A.

Received: October 18, 2010
Revised: December 19, 2010
Accepted: January 20, 2011
Published online: February 24, 2010

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