Shared Transcriptional Control Strategies of Developmental Signaling Pathways In Vivo

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Summary: Using a systematic in vivo assay in *Drosophila*, we find that transcription factors regulated by seven highly conserved cell signaling pathways obey the same functional principles of target gene control, including "activator insufficiency" and "default repression"; these allow each signaling pathway to tightly restrict the expression of its targets while retaining the flexibility required to generate diverse expression patterns in response to a common signal.

Abstract

Developmental cell signaling pathways each generate a remarkable diversity of target gene expression patterns. How is this flexibility achieved? Here, we investigate the transcriptional control strategies of seven ancient and highly conserved signaling pathways (Notch, Hedgehog, Wnt, TGF-β, Jak/STAT, nuclear receptor, and RTK/Ras/Erk) in transgenic *Drosophila*. We find that transcription factors regulated by these diverse pathways use a common set of functional principles in vivo, including an inability to generate a response to signaling without cooperating activators ("activator insufficiency"), and the capacity to directly repress target genes in the absence of signaling ("default repression"). Our observations include the first in vivo evidence for direct repression by *Drosophila* STAT and the ecdysone receptor.

Text

A small number of ancient cell signaling pathways control the vast majority of conditional cell fate decisions during animal development, primarily by regulating the activity of transcription factors, which in turn direct the expression of pathway target genes. Each pathway has many target genes, activated in diverse and often non-overlapping expression patterns. Such versatility allows a single pathway to be re-used many times, with different cell fate outcomes in each context. Without this ability to elicit multiple target gene expression patterns, each signaling pathway would be limited to a single developmental role, with no ability to acquire new functions. But how does a signaling pathway ensure that each signaling event activates only the correct subset of its target genes?

We will use the term "signaling pathway response elements" (SPREs) to refer to binding sites in DNA for signal-regulated transcription factors. In a recent review article (*1*), we proposed that many signaling pathways may use three shared transcriptional regulatory strategies to control expression of their various target genes. First, some published evidence suggests that SPREs, when taken out of their normal sequence context in cis-regulatory modules, are often unable to generate a transcriptional response to signaling in vivo. We have called this property "activator insufficiency" (*1*). Second, through synergistic interactions with non-signal-regulated activators ("cooperative activation"), SPREs can overcome their insufficiency and drive high levels of target gene expression. However, these non-signal-regulated, cell type- or tissue-specific ("local") activators are often sufficient to activate target genes, even in the absence of signaling. Hence the third shared strategy, "default repression" (*1*), or

repression by signal-regulated transcription factors in the absence of signaling, which ensures that target genes are turned off where there is no signal, even if local activators are present.

These regulatory properties, taken together, may explain the ability of pathways to limit the expression of each target gene to the appropriate developmental context(s). However, experimental support for the universality of these strategies is limited and incomplete. Cooperative activation has been documented for many signal-regulated factors (*1*), but repression is much less thoroughly studied, and in some pathways no direct in vivo evidence for default repression exists. As for activator insufficiency, although enhancer mutation studies frequently indicate that SPREs require the help of other inputs to mediate a transcriptional response to signaling, few direct in vivo tests of the function of isolated SPREs have been reported (*1*). Furthermore, these in vivo data are contradicted by a wealth of transient transfection experiments demonstrating that reporter constructs containing multimerized SPREs are readily activated in cultured cell lines upon pathway stimulation, resulting in ambiguity as to the true nature of transcriptional responses to signaling.

Here, we present the results of a series of standardized reporter transgene experiments in *Drosophila*, designed to examine the transcriptional regulatory behavior of seven major signaling pathways (Notch, Hedgehog, Wingless/Wnt, Jak/STAT, Dpp/TGF-β, RTK/Ras/Erk, and the ecdysone receptor, a well-studied member of the nuclear hormone receptor family), under normal conditions, in a variety of developmental signaling contexts. Reporter transgenes were constructed as shown in Fig. 1A. In each experiment, we placed four SPREs for a given pathway (e.g., four Suppressor of Hairless n binding sites in the case

of Notch signaling), separated by ~20-bp spacers, upstream of a TATA-containing minimal promoter driving the *lacZ* reporter gene in insulated P-element transformation vectors (2). All SPREs used are known high-affinity transcription factor binding sites, identified from signal-regulated enhancers and/or from in vitro binding assays (2). We also combined SPREs with binding sites for the activator protein Grainyhead (Grh), an approach devised by Furriols and Bray (3) to study Notch signaling. Grh is a useful stand-in for a non-signal-regulated "local" activator in these experiments for two reasons: First, multimerized Grh sites are sufficient for gene activation (ref. 3; Fig. 1B); second, Grh is expressed in the late embryonic and larval ectoderm, including imaginal discs (3), and therefore is present in cells responding to all seven pathways being tested. By observing the expression of these reporters in wild-type animals, we can determine the transcriptional responsiveness of SPREs to endogenous pathway signaling, with all pathway components present and active at biologically normal levels.

Transcriptional responses to Notch, Hedgehog, and Wnt signaling. Target gene regulation by the Notch pathway is mediated by the transcription factor Su(H) (and its homologs CBF1 and Lag-1), which acts as an activator during Notch signaling and a repressor in the absence of signaling (4). A construct containing four Su(H) binding sites [4xSu(H)-*lacZ*] is insufficient for reporter gene activation, even in cells receiving high levels of Notch signaling, such as the presumptive wing margin and proneural clusters of the wing imaginal disc (Fig. 1C). These results differ from those of Furriols and Bray (3), who found that Su(H) sites drive expression along the wing margin; however, their reporter constructs contained sequences known as Su(H) paired sites

(SPSs), which are characterized by a particular orientation and spacing of Su(H) sites and include additional conserved flanking sequences (*5*). Our 4xSu(H)-*lacZ* construct, which contains individual sites placed in tandem, has no such activity (Fig. 1C), although it is active in socket cells of mechanosensory bristles (ref. 1; data not shown); these cells, uniquely, display signal-independent activation by Su(H) and express very high levels of Su(H) protein (*6*). To exclude the possibility that the spacing of Su(H) sites in this construct fortuitously precludes activation, we added 7 bp to the spacing between the sites, with no effect on Notch responsiveness (not shown).

When Grh sites are placed upstream of Su(H) sites, both cooperative activation (at sites of Notch signaling) and active repression (in regions without signaling) are observed in wing discs (ref. 3; Fig. 1D; cf. panel B). In late-stage embryos, likewise, 3xGrh+4xSu(H)-*lacZ* [but not 4xSu(H)-*lacZ*] acts as a Notch pathway reporter, with Su(H) exhibiting both cooperative activation and default repression (Fig. 1C,D). This Notch-responsive construct also acts as a positive control for 4xSu(H)-*lacZ*, since the spacing, orientation, distance from the promoter, and flanking sequences of the Su(H) sites are identical in both constructs; we therefore conclude that 4xSu(H)-*lacZ*, though unable to respond to Notch signaling, contains functional Su(H) binding sites.

Hairless is an adaptor protein that recruits the co-repressors Groucho and CtBP to Su(H), and thus has a fundamental role in default repression by this factor (*7*). In flies with reduced Hairless levels, default repression of 3xGrh+4xSu(H)-*lacZ* is indeed diminished, but interestingly, 4xSu(H)-*lacZ* still fails to be activated (Fig. S1).

4xCi-*lacZ* contains four binding sites for Cubitus interruptus (Ci), the Gli-family transcription factor regulated by the Hedgehog (Hh) signaling pathway. Ci acts as a signal-dependent transcriptional switch (*8*). 4xCi-*lacZ* activity is absent or very weak at sites of Hh signaling (Fig. 1E). 3xGrh+4xCi-*lacZ*, by contrast, is a robust reporter of signaling, with strong cooperativity between Ci and Grh in Hh-responding cells (Fig. 1F, arrows), and potent default repression by Ci in non-responding cells (arrowheads, cf. panel B). 3xGrh+4xCi-*lacZ* also appears to be weakly active in some cells of the peripodial membrane (Fig. 1F), a site of expression of the Hh ligand (*9*). Ci gives the strongest cooperative activation of all factors tested in this study, possibly because the Ci sites used were defined by in vitro selection (*10*), which biases for the highest-affinity sites, rather than being taken from a native enhancer.

dTCF/Pangolin acts as a Wnt-dependent transcriptional switch in the *Drosophila* embryo, although it has recently been proposed that dTCF does not have a default repression function in imaginal discs (*11*). 4xTCF-*lacZ* is not detectably activated in embryos or imaginal discs, even at sites of high-level Wnt signaling (Fig. 1G, arrows). When combined with Grh sites, however, dTCF sites respond to Wnt signaling, though not in all Wnt-responding cells (Fig. 1H). Reporter gene expression is also observed in peripodial membrane cells (Fig. 1H), which express the Wnt ligand Wingless (*9*). We find that dTCF mediates powerful repression in both embryos and imaginal discs (Fig. 1H; cf. panel B).

The nuclear hormone receptor and Jak/STAT signaling pathways. The ecdysone receptor (EcR), a member of the nuclear receptor superfamily, functions as a heterodimer with the *Drosophila* RXR homolog, Ultraspiracle (Usp) (*12*). The hormone ecdysone is produced at high levels late in the third larval

instar, activating ecdysone-inducible genes via ecdysone response elements (EcREs). Circumstantial evidence for default repression via EcREs is very strong: EcR is known to interact with the co-repressor SMRTER (*13*); genetic experiments suggest that EcR/Usp may repress at least one target gene, perhaps directly (*14*); and EcREs can confer repression in transfected cell lines in the absence of hormone (*15*). However, EcREs have never been shown to mediate direct repression in vivo. Since the ecdysone pathway is temporally rather than spatially regulated, we examined mid- and late third-instar imaginal discs carrying 4xEcRE-*lacZ* to compare the activity of EcR in the absence and presence of ecdysone, respectively (Fig. 2A-C). While EcREs alone are unresponsive to hormone in vivo (Fig. 2B), they mediate strong default repression (Fig. 2C, bottom panel; cf. panel A) when combined with Grh sites. 3xGrh+4xEcRE-*lacZ* shows elevated expression relative to 3xGrh-*lacZ* in the wing pouch (Fig. 2C, top panel, arrow), a region of elevated EcR expression (*16*). However, we do not observe broad and robust cooperativity between EcRE and Grh.

The Janus kinase/Signal transducer and activator of transcription (Jak/STAT) pathway is perhaps the least extensively studied of the seven pathways examined here. Although two vertebrate STATs have been shown to interact physically with co-repressors (*17, 18*), no direct in vivo evidence exists in any organism for either default repression by STATs or insufficiency of STAT binding sites for activation. In *Drosophila*, the Jak/STAT pathway ligand Unpaired (Upd) is expressed at the posterior margin of the eye imaginal disc and in a complex pattern in embryos (*19, 20*). We find that 4xSTAT-*lacZ* fails to respond to Jak/STAT signaling in vivo (Fig. 2D, arrows). By contrast, 3xGrh+4xSTAT-*lacZ* is activated in embryos in a pattern similar to that of *upd*

transcript accumulation (Fig. 2E,F, bottom panels), suggesting that this construct acts as a Jak/STAT pathway reporter. In the eye disc, 3xGrh+4xSTAT-*lacZ* is activated at the posterior margin, a known site of Upd protein accumulation (*20*), though we were unable to detect *upd* transcript in these cells by RNA in situ hybridization (Fig. 2E,F, middle panels).

We also observed 3xGrh+4xSTAT-*lacZ* activity in specific regions of the wing hinge anlage in the wing imaginal disc (Fig. 2E, top panel). Although Upd expression in the wing has not been described, the *upd* gene is also known as *outstretched*, so named because a partial loss of function results in outstretched wings. This strongly suggested that *upd* is expressed in the developing wing hinge. Indeed, we found that *upd* transcript does accumulate in regions of the wing hinge primordium where our reporter is activated (Fig. 2F, top panel).

In addition to the patterned activation of 3xGrh+4xSTAT-*lacZ* observed in these experiments, the strong default repression mediated by *Drosophila* STAT in the absence of pathway signaling (Fig. 2E, arrowheads; cf. Fig. 1B) is also striking. This represents the first in vivo evidence for direct repression by a STAT in any organism. Thus, *Drosophila* STAT appears to exhibit all three proposed properties of signal-regulated transcription factors: activator insufficiency, cooperative activation, and default repression.

TGF-β and RTK/Ras/Erk signaling. In contrast to the Type I signaling pathways described above, in which the same transcription factor mediates both activation and default repression, we have defined Type II pathways as those in which two dedicated factors, an activator and a repressor, are both regulated by signaling (*1*). We cited two examples of this type of pathway in *Drosophila*: the Dpp/TGF-β pathway, in which Mad is an activator and Brinker (Brk) a

repressor; and the Ets transcription factors regulated by RTK/Ras/Erk signaling, of which Pointed (Pnt) is an activator and Yan/Aop is a repressor. In both cases, signaling stimulates the activator while inhibiting the repressor, and the activator and repressor commonly have overlapping binding sites in pathway target genes (*1*). We tested SPREs consisting of overlapping binding sites for Mad and Brk, or for Pnt and Yan, in vivo (Fig. 3). Mad/Brk sites are insufficient to respond to Dpp signaling in the eye imaginal disc and along the anterior/posterior compartment boundary of the wing disc, as well as in the embryo (Fig. 3A, arrows). However, we do detect 4xMad/Brk-*lacZ* expression in the embryonic amnioserosa and (very faintly) in part of the peripodial membrane of wing discs (Fig. 3A), both sites of expression of the Dpp ligand. Brk, which has been shown to represses some Dpp target genes, provides strong default repression in 3xGrh+4xMad/Brk-*lacZ* animals (Fig. 3B, red arrowheads), while Mad cooperates with Grh in regions of high Dpp signaling (Fig. 3B, arrows).

The *pnt* gene encodes two Ets-domain transcription factors, PntP1 and PntP2, by alternative splicing. We will refer to both isoforms (which have the same DNA-binding domain) aggregately as Pnt. Pnt is activated by RTK signaling in glia of the embryonic CNS, as well as during wing vein and retinal development. 4xPnt/Yan-*lacZ* is not activated in imaginal discs, although expression is observed in a subset of the embryonic CNS (Fig. 3C). 3xGrh+4xPnt/Yan-*lacZ* is expressed in the wing disc in a pattern suggestive of wing vein development, as well as in the eye disc posterior to the morphogenetic furrow (MF) (Fig. 3D, arrows). This reporter is expressed in a complex embryonic pattern, including sensory organs (Fig. 3D, black arrowheads). No repression by Yan is observed in the eye disc posterior to the MF, possibly due to

perduring β-galactosidase left behind by reporter activation in the advancing MF. However, repression can be seen in the wing disc and, most strikingly, in the embryo (Fig. 3D, red arrowheads; cf. Fig. 1B). Taken together, our results with Mad/Brk and Pnt/Yan sites suggest that SPREs for Type II pathways behave similarly to those for Type I pathways (that is, activator insufficiency, cooperative activation, and default repression all occur), but that activator sufficiency is observed in certain cell types. This limited sufficiency may reflect the fact that in Type II pathways, where activation and repression are mediated by separate (and separately regulated) factors, it is possible for signal-independent activation to occur in those territories that lack an SPRE-binding repressor species. This might be particularly true in the case of 4xPnt/Yan-*lacZ*: the PntP1 isoform is a constitutive activator, and does not require activation by RTK/Ras/Erk signaling (*21, 22*).

A synthetic signal-regulated enhancer. One of the best-studied examples of cooperative activation by a signal-regulated activator occurs in proneural cluster cells of imaginal discs, where the Notch-responsive factor Su(H) directly cooperates with the proneural bHLH activators Achaete and Scute (Ac/Sc) to activate several genes of the *Enhancer of split* gene complex [E(spl)-C] (*5, 21, 22*). In an attempt to build a synthetic signal-regulated enhancer, we have combined Su(H) and Ac/Sc binding sites in a transgenic reporter construct. Both Su(H) and Ac/Sc are capable of strongly activating target genes via a single binding site, in the context of a native enhancer (*23-25*). Nevertheless, our 2xSu(H)+2xAc/Sc-*lacZ* construct is not activated in proneural cluster cells (Fig. 4A). Other configurations of Su(H) and Ac/Sc sites similarly fail to recapitulate E(spl)-C gene expression patterns (*26*). Only when binding sites for the activator

Grh are added to the reporter construct does a proneural cluster pattern emerge (Fig. 4B). Key to the cell-type-specific expression of this synthetic enhancer is default repression by Su(H), which prevents Grh-mediated activation in cells lacking Notch signaling (Fig. 4B, arrowhead; cf. Fig. 1B). Note that Grh is not a known regulator of any Su(H) or Ac/Sc target gene. This result suggests that additional transcriptional activators besides Su(H) and Ac/Sc may be required for the activation of their target genes, and that simply combining inputs from a signal-regulated activator and a local activator may not be sufficient to create a signal-responsive enhancer, as has been proposed (*27*). However, it is also possible that the particular orientation and/or spacing of Su(H) and Ac/Sc sites that would create a functional synthetic enhancer has not yet been tried, and that the addition of Grh sites merely serves to compensate for this improper arrangement.

Switching the tissue specificity of a pathway target gene. The *E(spl)m6* gene is activated by Notch/Su(H) and the mesodermal bHLH activator Twist in adult muscle precursors of the embryo and wing disc; a 524-bp promoter fragment containing three Su(H) sites and two Twist-type E boxes recapitulates this expression (*28*) (Fig. 4C, arrowhead). By converting the E boxes from those that bind Twist to those that bind the proneural bHLH activators Ac/Sc, we switched the cell-type specificity of this enhancer from Notch-responding mesodermal cells (muscle precursors) to Notch-responding ectodermal cells (proneural clusters) (Fig. 4D, arrowheads). This successful result in a native enhancer demonstrates the critical importance of local activators for determining the expression specificity of pathway target genes, and suggests the evolutionary

flexibility inherent in cis-regulatory modules that utilize cooperative inputs from local and signal-regulated factors.

Sufficiency of local activators. We have shown here that, in most cases, signal-regulated activator proteins are unable to elicit transcriptional activation in vivo without cooperative partners. Are non-signal-regulated "local" activator proteins similarly insufficient? We have demonstrated previously that four Ac/Sc binding sites are sufficient to generate a proneural cluster expression pattern (Fig. 4E; ref. 1). Similarly, we find that four Twist-type E boxes drive gene expression in adult muscle precursors of the wing disc (Fig. 4F). Taken together with the transcriptional sufficiency of Grh (Fig. 1B), this small sample is consistent with the idea that local activators are typically not subject to the same restrictions on activator potency that apply to signal-regulated activators.

Shared transcriptional regulatory principles. We have employed a standardized reporter assay to test the transcriptional regulatory properties of SPREs in transgenic *Drosophila*. We believe there are several important advantages to this systematic experimental approach. First, it permitted us to compare the different signaling pathways and their SPREs side-by-side using the same controlled method. Second, the responses of the different SPREs were examined in wild-type animals under normal conditions of pathway signaling. Third, the minimal nature of the reporter constructs simplifies the task of interpretation and strengthens the possible conclusions, as compared to experiments with complex native enhancer modules. Thus, we are able to attribute the transcriptional regulatory properties we observed specifically to known signal-regulated factors, either alone or in interaction with Grh.

The overall results of these analyses are summarized qualitatively in Figure 5. Our most striking findings concern the transcriptional regulatory properties of "activator insufficiency" and "default repression". All seven of the tested pathways display clear evidence of utilizing both principles in vivo.

Multimerized high-affinity REs for the Notch, Wnt, EcR, and Jak/STAT pathways were wholly unable to activate transcription in response to normal pathway signaling (Fig. 5). Two lines of evidence strongly suggest that such negative results are not artifacts of our choice of SPRE sequences or of the design of the constructs (e.g., spacing). First, the finding that the same binding sites in the same configuration support cooperative activation and/or default repression when combined with Grh sites indicates that the chosen SPREs are indeed able to function in vivo to mediate the action of the cognate transcription factor. Second, in the case of Su(H) binding sites (Notch SPREs), we have found that a 7-bp increase in site spacing fails to make 4xSu(H)-*lacZ* responsive to Notch. In the case of the remaining pathways (Hedgehog, TGF-β, and RTK/Ras/Erk), varying degrees of completeness of activator insufficiency are observed. For Hedgehog, only very weak sufficiency is apparent in a small subset of signal-responding cells. SPREs for the Dpp/TGF-β pathway are sufficient only in two unusual squamous epithelia, the embryonic amnioserosa and the peripodial membrane associated with imaginal discs. Finally, Pnt/Yan sites are sufficient to direct reporter gene expression in a small subset of the embryonic CNS. A priori, one might have expected some degree of sufficiency to be exhibited by 4xPnt/Yan, since *Drosophila* has six other genes that encode Ets-family transcription factors that in principle could activate transcription via these sites wherever repressor

proteins like Yan (Aop) are absent. None of the seven pathways was able to strongly and consistently activate a transgene containing multimerized response elements, contrasting starkly with transient transfection experiments in cultured cells, in which all seven pathways generate robust transcriptional responses via multimerized SPREs (1).

The extent to which the different SPREs support the transcriptional property of "default repression" is equally striking (Fig. 5). Particularly noteworthy is the potent repression conferred by binding sites for dTCF, EcR, and STAT. In contrast to the conclusion from a recent study (*11*), we observe robust repression mediated by dTCF sites in the wing imaginal disc, suggesting that dTCF does function as a default repressor in this setting. Our experiments clearly associate temporal control of gene expression by the hormone ecdysone with default repression via EcREs. Finally, STAT sites had not previously been known to mediate default repression in any organism. Collectively, our results are consistent with a general role for default repression in signal-regulated gene expression in vivo.

Although default repression and cooperative activation were evident for every pathway studied, we sometimes observed spatial variations in the strength of these properties, as well as differences in overall intensity from one pathway to another. While these differences may be biologically significant, they may instead reflect different optima for binding site spacing, or different levels of interactivity between our panel of transcription factors and Grh, which is not known to cooperate with any of these proteins during normal development. The fact that all seven pathways evinced both positive and negative interactions with Grh attests to a high degree of flexibility among these signal-regulated factors.

Overall, these systematic experiments strongly support the conclusion that the seven ancient developmental signaling systems considered here all make use of the three transcriptional regulatory principles of activator insufficiency, cooperative activation, and default repression to control the expression of their target genes in vivo. These shared properties of signal-regulated transcription factors may explain the remarkable ability of signaling pathways to activate expression of multiple target genes in diverse patterns, since they insure that target gene activity is tightly restricted to those cells that both receive pathway signaling *and* express appropriate local activators. Such elegant control grants signaling pathways tremendous evolutionary flexibility in acquiring new target genes and new functions in development and physiology.

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29. R. Bodner characterized the wild-type 524-bp *E(spl)m6* promoter fragment shown in Fig. 4C. We thank D. Harrison for providing an *upd* cDNA clone.

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Figure Legends

Fig. 1. Response elements (REs) for the "Type I" signaling pathways Notch, Hedgehog, and Wnt are largely insufficient to activate transcription in vivo, and exert "default repression" in the absence of signaling. Arrows indicate regions of active pathway signaling; red arrowheads denote repression via SPREs in cells that do not receive signaling. (**A**) Design of transgenic pathway reporters. In (**B**)-(**H**), the top panel shows a wing imaginal disc from a late third-instar larva, the middle panel shows an eye imaginal disc, and the bottom panel shows a stage-16 embryo. Imaginal discs (dorsal to right, posterior to top) are stained with X-gal to reveal β-galactosidase activity, and embryos (dorsal to top, anterior to left) are stained for *lacZ* RNA by in situ hybridization. (**B**) 3xGrh-spacer-*lacZ* is activated by Grh in all cuticle-forming tissues, including imaginal discs and late embryonic ectoderm. (**C**),(**D**) Su(H) binding sites (Notch pathway REs). See text for details. (**E**),(**F**) Ci binding sites (Hedgehog pathway REs). (**G**),(**H**) TCF binding sites (Wingless/Wnt pathway REs). wm, wing margin anlage; pc, proneural cluster; pm, peripodial membrane.

Fig. 2. Activator insufficiency and default repression revealed by ecdysone and Jak/STAT REs. Arrows indicate sites of known pathway signaling; red arrowheads denote SPRE-mediated repression in regions of pathway inactivity. (**A**)-(**C**) EcR/Usp binding sites (EcREs). Top panels show wing imaginal discs from larvae at late third instar, when an ecdysone pulse occurs; bottom panels show wing discs from mid-third-instar larvae, prior to the ecdysone pulse. (**D**),(**E**) D-STAT binding sites (Jak/STAT pathway REs). (**F**) Expression of

unpaired in imaginal discs and embryos, assayed by RNA in situ hybridization. See text for details.

Fig. 3. REs for the "Type II" pathways Dpp/TGF-β and RTK/Ras/Erk reveal insufficiency and default repression, with exceptions to insufficiency in specific cell types. Arrows indicate sites of known pathway signaling; red arrowheads denote SPRE-mediated repression in regions of pathway inactivity. (**A**),(**B**) Mad/Brinker binding sites (Dpp/TGF-β pathway REs). (**C**),(**D**) Pointed/Yan binding sites (RTK/Ras/Erk pathway REs). In (**A**), the third panel shows a dorsal view of a stage-5 embryo; the fourth panel shows a lateral view of a stage-15 embryo. In (**B**), the third and fourth panels show lateral and ventral views, respectively, of a stage-13 embryo. In (**C**), the third panel shows a ventral view of a stage-12 embryo; the fourth panel shows an optical horizontal section of a stage-16 embryo. Only the embryonic ventral midline staining in (**C**) is due to Pnt/Yan sites; the rest is due to vector sequences. In (**D**), the third panel shows an optical horizontal section of a stage-14 embryo; the fourth panel shows a dorsal view of a stage-16 embryo. pm, peripodial membrane; as, presumptive amnioserosa; vm, ventral midline; mo, mechanosensory organ.

Fig. 4. Non-signal-regulated "local" activators are often sufficient to activate transcription, and can control the cell-type specificity of signal-regulated cis-regulatory modules. (**A**) Two Su(H) binding sites combined with two Ac/Sc sites are insufficient for gene activation in proneural cluster cells of imaginal discs. (**B**) Adding three Grh sites to this construct allows activation in proneural cluster

cells (arrows); strong default repression by Su(H) is also observed (red arrowhead; cf. Fig. 1A). (**C**) A 0.5-kb fragment of the *E(spl)m6* promoter drives GFP expression in muscle precursors of imaginal discs (arrowhead) in direct response to Notch/Su(H) and Twist. (**D**) Converting the two Twist binding sites to Ac/Sc sites switches the specificity of reporter gene expression from muscle precursors to proneural cluster cells, where both Notch and Ac/Sc are active (arrowheads). (**E**) Four Ac/Sc binding sites are sufficient for gene activation in cells expressing Ac/Sc (arrows). (**F**) Four Twist binding sites are sufficient for activation in Twist-expressing muscle precursors (arrow).

Fig. 5. Summary of results of standardized transgenic reporter assays. The cartoons at right illustrate the three transcriptional regulatory properties being assayed. ++: Property is clearly observed. +: Property is observed, but less strongly or less broadly. +/-: Property is not conclusively observed at most sites. SPRE, signaling pathway response element. Gray circle represents a signal-regulated transcription factor bound to an SPRE; green oval, a transcriptional co-activator protein; red oval, a co-repressor; blue hexagon, a local activator.

Figures

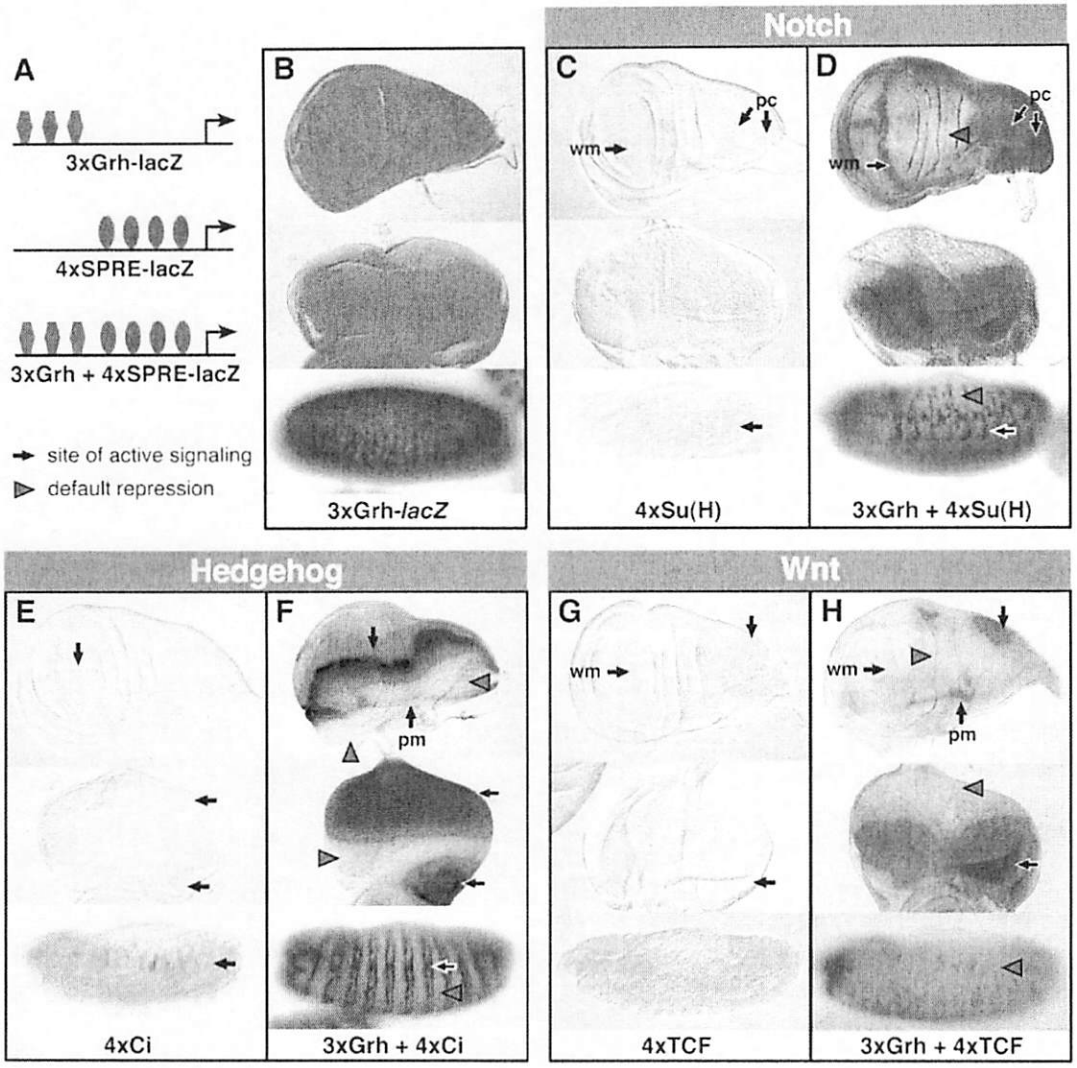

Figure 1

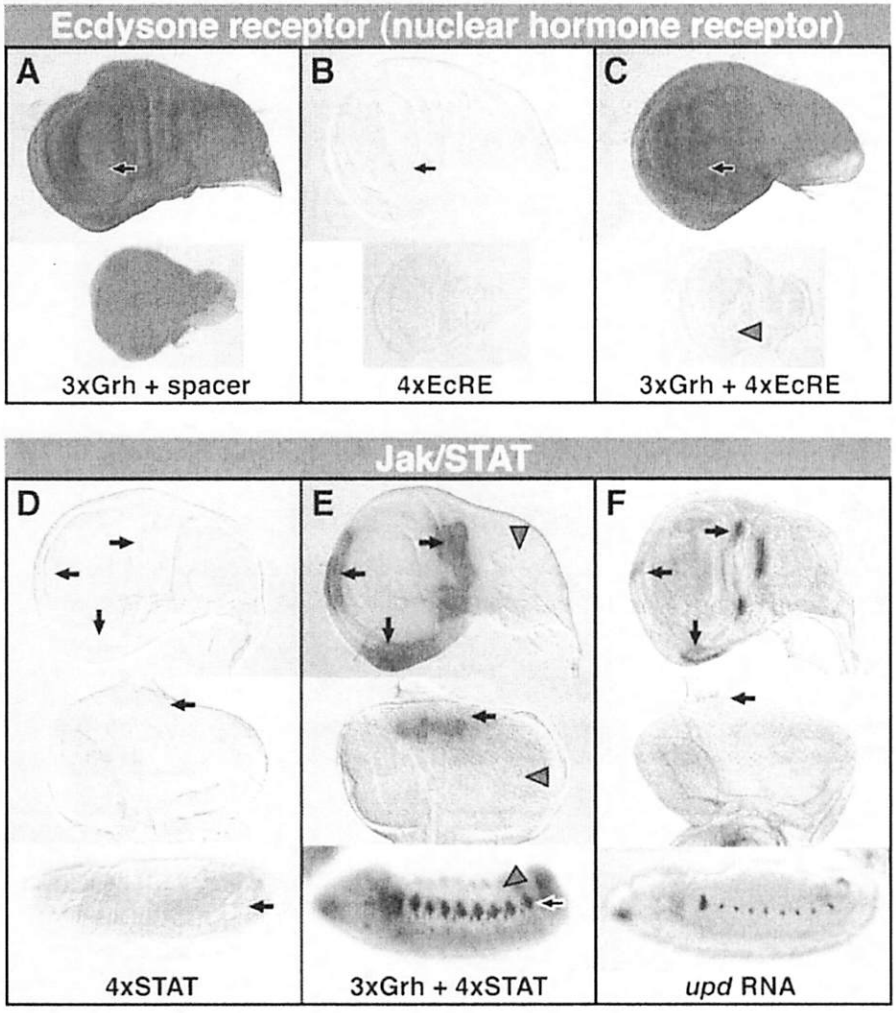
Ecdysone receptor (nuclear hormone receptor)
A
B
C
3xGrh + spacer
4xEcRE
3xGrh + 4xEcRE
Jak/STAT
D
E
F
4xSTAT
3xGrh + 4xSTAT
upd RNA

Figure 2

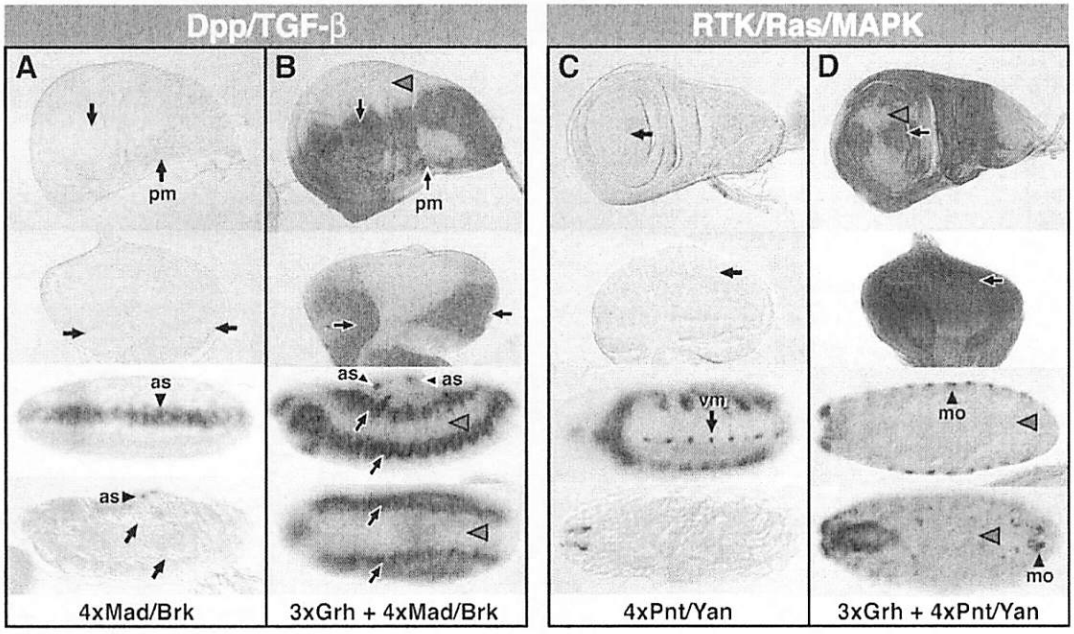
Dpp/TGF-β
RTK/Ras/MAPK
A
B
C
D
pm
pm
as
as
as
as
vm
mo
mo
4xMad/Brk
3xGrh + 4xMad/Brk
4xPnt/Yan
3xGrh + 4xPnt/Yan

Figure 3

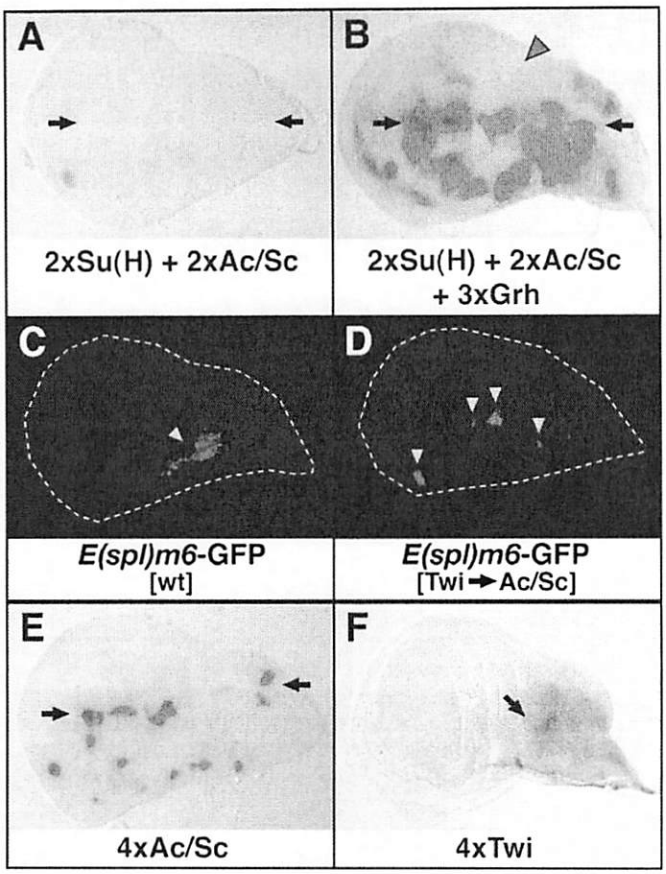
A
2xSu(H) + 2xAc/Sc
B
2xSu(H) + 2xAc/Sc
+ 3xGrh
C
E(spl)m6-GFP
[wt]
D
E(spl)m6-GFP
[Twi→Ac/Sc]
E
4xAc/Sc
F
4xTwi

Figure 4

	Notch	Hedgehog	Wnt	EcR	Jak/STAT	TGF-β	RTK/Ras/Erk	
Activator Insufficiency	++	+	++	++	++	+	+	
Cooperative Activation	++	++	+/-	+/-	++	++	+/-	
Default Repression	+	++	++	++	++	++	++	

Figure 5

SUPPORTING ONLINE MATERIAL

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Materials and Methods

Figure S1

Table S1

Materials and Methods

***Drosophila* transgenes.** Synthetic promoter constructs were assembled in the insulated *lacZ* reporter vector pH-Pelican (*1*), which contains a minimal TATA-bearing promoter from the *Hsp70* gene. Transcription factor binding site sequences are shown in Supporting Table S1. Binding sites were separated by spacers derived from the coding sequence of the *E. coli* chloramphenicol acetyltransferase (CAT) gene. Double-stranded oligonucleotides containing two or three binding sites each were serially ligated into the vector upstream of the promoter. In all cases, binding sites were arranged in the same orientation relative to the promoter, with 26-29 bp separating the 5'-most bases of each pair of sites. For example, the 4xSu(H)-*lacZ* promoter has the following sequence, with Su(H) sites in capitals and the TATA sequence underlined:

...gctagcagtactcgaggcTGTGGGAAtactcagccaatccctgagCGTGG

GAAgacagttttgattttcgaggcTGTGGGAAtactcagccaatccctga

gCGTGGGAAgacagttttgattttcgaggagcgccggagtataaa...

In Grainyhead (Grh) site-containing constructs, the 3'-most Grh site is 24-26 bp 5' (upstream) of the nearest binding site for another factor. For example, the 3xGrh-4xSu(H)-*lacZ* promoter has the following sequence, with Grh sites in underlined capitals, Su(H) sites in capitals, and the TATA sequence underlined:

...agatctaAACCGGTTatgcgagtctagacttggaAACCGGTTatgcgagt

ctagacttggaAACCGGTTatgcggccgctagcagtactcgaggcTGTGG

GAAtactcagccaatccctgagCGTGGGAAgacagttttgattttcgagg

cTGTGGGAAtactcagccaatccctgagCGTGGGAAgacagttttgattt

tcgaggagcgccggagtataaa...

3xGrh-spacer-*lacZ* includes a CAT spacer (in capitals) to maintain a constant distance between Grh sites and the promoter in all Grh experiments:

...agatctaAACCGGTTatgcgagtctagacttggaAACCGGTTatgcgagt

ctagacttggaAACCGGTTatgcggccgctagcagtactcgagTATGTTTT

TCGTCTCAGCCAATCCCTGGGTGAGTTTCACCAGTTTTGATTTTCGAGTA

TGTTTTTCGTCTCAGCCAATCCCTGGGTGAGTTTCACCAGTTTTGATTtt

cgaggagcgccggagtataaa...

The 524-bp *E(spl)m6* promoter fragment (which includes the native promoter and transcription start site) was ligated into the insulated eGFP-NLS reporter vector pStinger (*1*). The promoter fragment extends from -487 to +37, relative to the transcription start site. *E(spl)m6*-GFP[Twi->Ac/Sc] was created by a PCR sewing strategy which changed the following sequence, including two consensus Twist binding sites (in capitals):

...tggaCATGTGtccgcCACATGTGgca... to this sequence, containing two copies of an Ac/Sc binding site taken from the *E(spl)mα* enhancer (2) (in capitals):

...tGGCAGGTGTTCCTcagccggcGGCAGGTGgca...

Histochemistry. *lacZ* reporter gene expression in imaginal discs was visualized by assaying β-galactosidase activity as described (3). Animals carrying one copy of the reporter transgene were stained. Grh site-containing

constructs were stained at room temperature in 0.2% X-Gal for 30 minutes; constructs without Grh sites were stained for 90 minutes. Digoxigenin-labeled antisense RNA probes for *lacZ* and *upd* were prepared essentially as described (*4*). In situ hybridizations in embryos and imaginal discs were performed essentially as described (*5, 6*). GFP-expressing discs were fixed in 4% formaldehyde in PBS for 10 minutes at room temperature, mounted in 70% glycerol in PBS, and imaged in a confocal microscope.

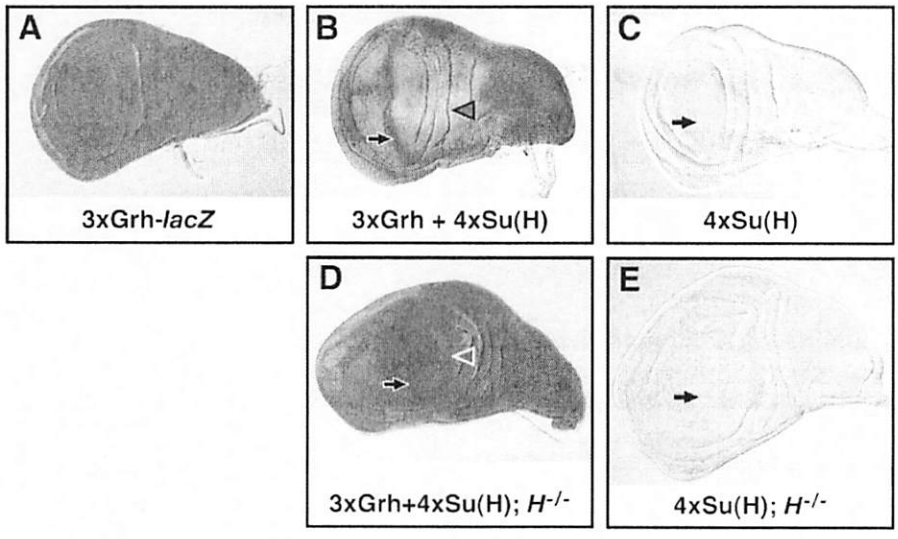
***Drosophila* genetics.** Discs in Fig. S1D,E are from H^2/H^{E31} larvae. H^2 is a strong hypomorphic allele of *Hairless* (*7*); H^{E31} is a null allele (*8*). H^2/H^{E31} animals die as early pupae.

SUPPORTING ONLINE MATERIAL (continued)

Legend for Supporting Figure

Fig. S1. Loss of the co-repressor/adaptor protein Hairless impairs default repression mediated by Notch response elements, but does not relieve activator insufficiency. Wing imaginal discs are stained and oriented as in Fig. 1. Arrows indicate the presumptive wing margin, a site of active Notch signaling, while red arrowheads indicate a region not receiving Notch signaling. Discs in (**A**)-(**C**) are taken from wild-type animals, while those in (**D**)-(**E**) are from flies lacking *Hairless* (*H*) gene function. (**A**) Three Grh binding sites drive reporter gene expression throughout the disc. (**B**),(**D**) 3xGrh+4xSu(H)-*lacZ* is significantly repressed in cells not receiving Notch signaling in wild-type, but not *H* mutant, discs. (**C**),(**E**) Four Su(H) sites are not sufficient to respond to Notch signaling in either wild-type or *H* mutant discs.

Supporting Figure S1



Supporting Table S1

Table S1. Transcription factor binding sites used in this study. Flanking bases used in reporter constructs are shown in lowercase. Full promoter sequences are available upon request.

Factor(s)	Site Sequence	Source	Reference
Grh	AACCGGTT	*Ddc* enhancer	(*9*)
Su(H)	gcTGTGGGAAta	*Su(H)* enhancer	(*10*)
	agCGTGGGAAga	*Su(H)* enhancer	(*10*)
Ci	TGGGTGGTC	Gli consensus	(*11, 12*)
dTCF/Pan	CTTTGATCTcg	*Ubx* enhancer	(*13*)
	ccGTTTGATGT	*Ubx* enhancer	(*13*)
EcR	AAGTGCATTGAACCC	*Hsp27* promoter	(*14*)
D-STAT	TTTCCCGGAAAt	In vitro selection	(*15*)
Mad/Brk	GCGCCGGCGCTg	*Ubx* enhancer	(*16*)
Pnt/Yan	gTCAGGAAACag	*D-Pax2* enhancer	(*17*)
	tACCGGAAAAca	*D-Pax2* enhancer	(*17*)
Ac/Sc	ggGCAGGTGTTtc	*E(spl)mα* enhancer	(2)
Twi	CACATGTG	*Mef2* enhancer	(*18*)

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