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IN VIVO ANALYSIS OF THE MOLECULAR MECHANISM OF REPRESSION OF THE *DROSOPHILA* LONG-RANGE REPRESSOR HAIRY

By

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ABSTRACT

IN VIVO ANALYSIS OF THE MOLECULAR MECHANISM OF REPRESSION OF THE *DROSOPHILA* LONG-RANGE REPRESSOR HAIRY

By

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The *Drosophila* Hairy protein belongs to a widely conserved class of transcriptional repressors called Hairy and Enhancer-of-Split (HES) proteins that have important roles in embryogenesis and cell fate determination. HES family members are characterized by a conserved basic helix-loop-helix DNA binding domain, an Orange domain, and a C-terminal WRPW motif that binds the TLE/Groucho corepressor. While enormous advances have been made in elucidating the biological functions of HES proteins, there is very little molecular understanding of the mechanism by which they repress transcription. To address this issue, Hairy repression was analyzed in the context of a highly defined *Drosophila* embryo system. The system consisted of embryos carrying a *lacZ* reporter transgene activated ubiquitously by the yeast Gal4 protein, the latter of which was expressed under control of the *daughterless* promoter. Uniform repression of the system was achieved by the heat shock induction of a LexA-Hairy fusion protein that can bind to and repress the reporter. Using embryo chromatin immunoprecipitation, I have analyzed the recruitment of activators, coactivators, repressors, corepressors, and chromatin modifications associated with Hairy repression. The results show that during repression activators and coactivators remain bound to the promoter, suggesting that Hairy does not work by blocking activator and coactivator recruitment. Chromatin immunoprecipitation analysis showed that during repression Groucho was recruited to the entire four kilobase coding region of the *lacZ* reporter. Groucho binding was accompanied by recruitment of

the histone deacetylase Rpd3, a known Groucho interacting protein, and a subsequent loss of H3 and H4 acetylation levels at the promoter and one kilobase downstream. Concomitant with the loss of histone acetylation levels, a two-fold increase in total histone H3 occupancy was seen at the promoter region, suggesting that Hairy repression is associated with chromatin remodeling. Given that previous studies had characterized Hairy as a long-range repressor capable of dominantly inhibiting distal elements, the results suggest a model wherein Groucho spreading and subsequent histone deacetylation can lead to long-range gene silencing. However, unlike previously characterized long-range silencing mechanisms that involve corepressor spreading, the repression observed in this system was transient.

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KEY TO ABBREVIATIONS

AS-C	:	Achaete-Scute Complex
bHLH	:	basic helix-loop-helix
bp	:	base pair
ChIP	:	Chromatin immunoprecipitation
CK2	:	Cyclin-dependent kinase 2
CtBP	:	C-terminal binding protein
CTD	:	C-terminal domain
Da	:	Daughterless
Dam	:	DNA adenine methyltransferase
DSP	:	Dithiobis(succinimidyl propionate)
E(spl)	:	Enhancer of split
Emc	:	Extramacrochaetae
eve	:	even-skipped
ftz	:	fushi-taratzu
Gro	:	Groucho
GTF	:	General transcription factor
HAT	:	Histone acetyltransferase
HDAC	:	Histone deacetylase
HES	:	Hairy and Enhancer of split
Hesr	:	Hairy and Enhancer of Split related
HLH	:	Helix-loop-helix
HMT	:	Histone methyltransferase
HS	:	Heatshock
kb	:	kilobase pair
Kni	:	Knirps
Kr	:	Krüppel
ORF	:	Open reading frame
Pc	:	Polycomb
PcG	:	Polycomb group
PCR	:	Polymerase chain reaction
PIC	:	Pre-initiation complex
PNS	:	Peripheral nervous system
Pol	:	Polymerase
rho	:	rhomboid
SAGA	:	Spt-Ada-Gcn5-acetyl transferase complex
Sir2	:	Silent information regulator 2
Slp1	:	Sloppy-paired 1
Su(Hw)	:	Suppressor of Hairy wing
TAF	:	TBP-associated factor
TBP	:	TATA binding protein
TLE	:	Transducin-like-enhancer-of-split
twi	:	twist
UAS	:	Upstream activating sequence

Chapter I

Literature Review

The purpose of this project is to elucidate the molecular mechanism of repression of the long-range repressor Hairy in the context of a *Drosophila* embryo. Hairy belongs to a widely conserved family of transcriptional repressors called Hairy and Enhancer-of-Split (HES) proteins that have important roles in development and in determining cell fate decisions. While a wealth of information on the function of HES proteins exists, relatively little is known about the underlying mechanism of repression [1, 2]. A difficulty in studying developmental regulation *in vivo* is that only a specific subset of genes are expressed in different tissues. Thus, in early *Drosophila* development only a fraction of the cells express Hairy [3]. In order to solve this problem, I created an *in vivo* system wherein a transcriptional switch homogenously regulates the expression of a *lacZ* reporter within the *Drosophila* embryo. Activation of the switch was achieved by the ubiquitous expression of the yeast Gal4 activator, while repression was brought about by the heatshock induction of Hairy fused to the DNA-binding bacterial LexA protein. Using this system, both the repressor and the activator could be tracked on the gene, as well as the associated cofactors and chromatin modifications. Furthermore, by manipulating the expression of Gal4 and LexA-Hairy, the system allowed the differentiation between activator- and repressor-specific effects.

In this chapter I will review the literature with a focus on placing Hairy repression in a developmental and mechanistic context. To that end, I will discuss the functional roles of Hairy and related proteins in regulating *Drosophila* and vertebrate development.

I will also discuss in general terms the different mechanisms of transcriptional repression and describe what is known about the mechanism of repression by HES proteins. Given that the repression mechanism studied here takes place in the context of a reporter system activated by Gal4, I will include a section on the mechanism of Gal4 activation.

The results of this study show several important aspects of Hairy repression. First, tethering Hairy to the reporter silences the gene yet it does not displace the activator or coactivator from the promoter (Chapter 2). Second, repression is associated with the binding of the Hairy corepressor Groucho to an extensive region encompassing several kilobases, suggesting that Groucho can spread throughout the gene (Chapter 3). Third, repression was accompanied by recruitment of the Rpd3 histone deacetylase and a subsequent loss of acetylated histones at the promoter (Chapter 3). Lastly, repression was not permanent as it was readily reversed upon degradation of LexA-Hairy with a corresponding increase in histone acetylation levels (Chapter 2 and 3).

Early developmental regulation in *Drosophila*

In recent years, great strides have been made in understanding how gene activity is regulated in eukaryotes. The use of unicellular model systems such as yeast has provided a wealth of information on the molecular mechanism of regulation [4]. However, much less is known about the mechanisms specifying the complex temporal and spatial patterns of gene expression in developing organisms. One of the difficulties in studying gene regulation in metazoans is that each different cell type requires a specific genetic program that leads to the activation and repression of different subsets of genes [5]. Thus, in order to elucidate the mechanistic details of transcriptional regulation in

multicellular organisms, it is necessary to take into account the tissue and developmental time frame in which it occurs.

The fruitfly *Drosophila melanogaster* has been the organism of choice for studying developmental regulation. Its relatively small genome and the abundance of genetic tools provide an ideal platform for the systematic analysis of individual genes, as well as for the study of gene networks. Another advantage is the rapid rate of *Drosophila* development [6]. In only 24 hours, a single fertilized egg develops into a larva composed of multiple different tissues and organs [6]. This rapid rate of development greatly reduces the time needed to generate and characterize different *Drosophila* lines. In addition, early development occurs in a scyncytial embryo containing only nuclei and no cellular membranes, simplifying transcription regulatory models by allowing intercellular signaling events to be ignored [6]. Multiple studies of *Drosophila* development have shown that most regulatory mechanisms are conserved in higher organisms, including humans [7].

Early *Drosophila* development is characterized by a hierarchical cascade of transcriptional factors that progressively refine the expression patterns of genes involved in the establishment of segment identity [8]. Initially, maternally deposited RNA at the anterior and posterior poles of the embryo creates wide gradients of the maternal genes (*bicoid*, *hunchback*, *caudal*, and *nanos*) that serve as a coordinate system for specifying positional information [8]. In turn, the maternal genes regulate the expression of the zygotically expressed gap genes (zygotic *hunchback*, *knirps*, *kruppel*, *giant*, *tailless*, *huckebein*), so-called because null mutations cause a "gap" phenotype wherein multiple contiguous segments of the larvae are missing [9]. Gap genes then function to specify the

seven-stripe pattern of the pair-rule genes (*even-skipped*, *fushi-tarazu*, *hairy*, *runt*, *paired*, *odd-skipped*, *odd-paired*, *sloppy-paired*), mutations of which cause the loss of every other segment [9]. Finally, pair-rule genes are involved in regulating the expression of the segment polarity genes (*wingless*, *hedgehog*, and *engrailed*), which are expressed in a series of 14 stripes and function to define the metameric segment boundaries [10].

Transcriptional repression in *Drosophila*

A common theme of *Drosophila* segmentation is that repressors play a critical role in establishing the boundaries of gene expression domains [11]. For example, formation of the seven stripe pattern of *even-skipped* (*eve*) involves the additive action of modular *cis* regulatory elements or "enhancers" that establish stripes 3/7, 2, 4/6, 1 and 5 respectively [12]. Initially, broadly expressed activators such as Bicoid, Hunchback, and Stat bind to these elements and activate expression throughout the embryo [8]. However, as development progresses, stripes become more defined as the gap genes bind to these modules and repress their activity in the interstripe regions [8]. Thus, formation of the *eve* stripe 2, involves activation in the anterior half of the embryo by Bicoid and Hunchback and repression by Giant and Kruppel at the anterior and posterior borders respectively [8, 11].

Despite the importance of repression in developmental regulation of eukaryotes, early studies focused mostly on the mechanism of transcriptional activation [13]. This focus was due to the idea that since only 7% of genes are transcribed at any one time in an eukaryotic cell, it is more cost effective to activate the genes needed than it is to repress the rest [13]. Thus, it was believed that most transcription factors should function

as activators [14]. In addition, repression was viewed simply as a disruption of the activation process [13]. However, with the discovery and characterization of many repressor complexes, it is now clear that repression plays a big part in transcriptional regulation and can involve multiple mechanisms [15, 16].

In *Drosophila*, repressors are often grouped into two functional classes depending on their range of activity [17]. Short-range repressors like Snail, Knirps, Kruppel, and Giant can disrupt the functions of activators or the promoter only if they are bound within 100-150 bp (Figure 1-1A) [18-21]. Such a limited range seems well suited to maintaining the enhancer autonomy of modular *cis* regulatory elements such as those found on *eve* [18]. In contrast, long-range repressors like Hairy and Polycomb (Pc) can silence multiple enhancers in a dominant fashion even when bound over 1 kb away from the nearest activator or promoter (Figure 1-1B) [22-24].

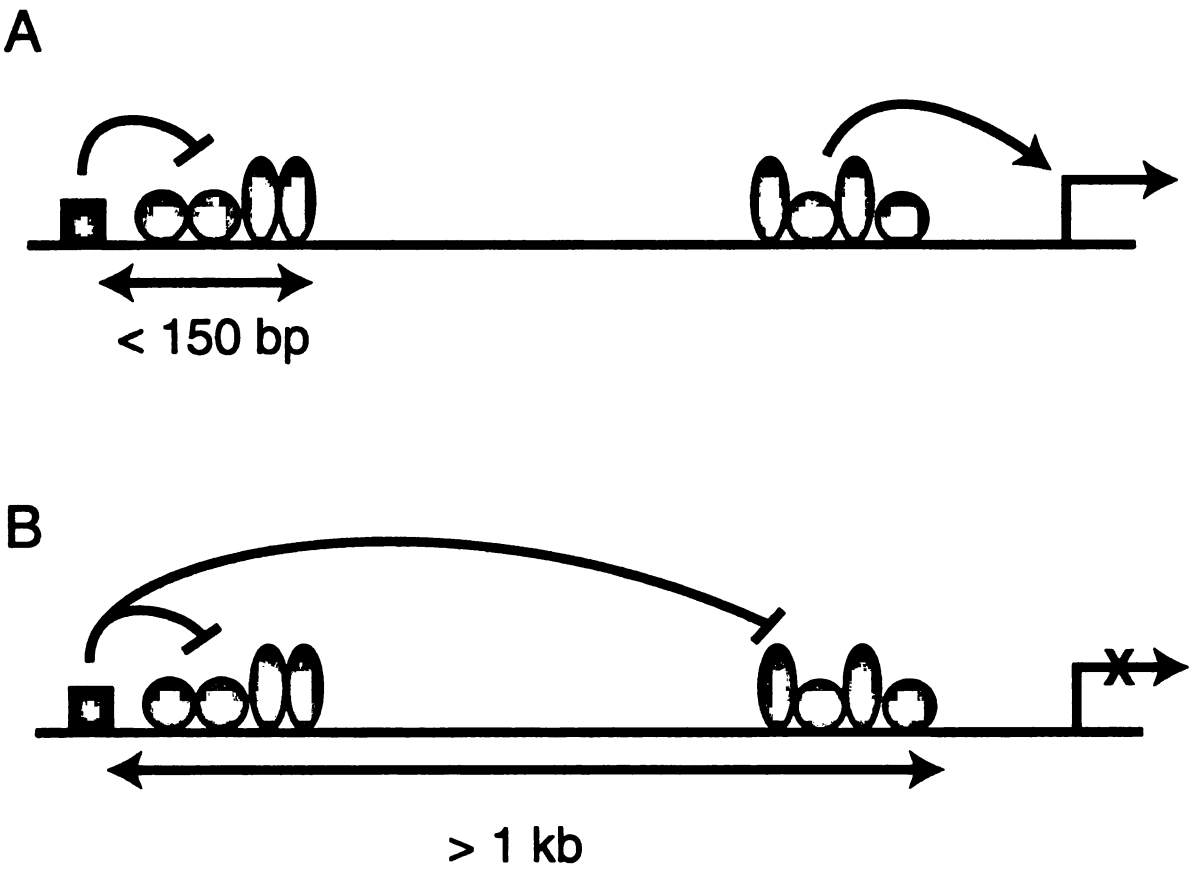

Figure 1-1. Long-range and short-range repression.

(A) Short-range repressors like Knirps, Snail, Giant, and Kruppel interfere with the function of activators that lie within 100-150 bp from the repressor. By repressing locally short-range repressors maintain enhancer autonomy. **(B)** Long-range repressors like Hairy can dominantly repress distant enhancers over distances of several kilobases. Repressors are represented by squares and activators by ovals. Images in this dissertation are presented in color.

Mechanisms of transcriptional repression

Transcriptional repressors can be characterized as being global or gene-specific. Global repressors typically function by sequestering or modifying components of the basal transcriptional machinery, such that it is not available for transcription, resulting in the down-regulation of genes that require that component [16]. For example, herpes virus infection can lead to aberrant phosphorylation of the Pol II CTD, resulting in a down-regulation of genes transcribed by Pol II [25]. Gene-specific repressors on the other hand regulate a much smaller subset of genes. This type of repression can be effected by disrupting the formation of an activator/DNA complex or by blocking their stimulatory interactions with the promoter (Figure 1-2A) [16, 26]. Alternatively, gene-specific repression can occur by interactions with the basal machinery at a particular promoter (Figure 1-2B) [16]. In addition, repressors can recruit chromatin modifying enzymes to locally alter the chromatin structure so that it becomes refractive to transcription (Figure 1-2C) [16]. Many gene-specific repressors contain DNA-binding domains that enable them to specifically bind to their targets [26]. In contrast, some repressors are recruited to genes via interaction with other DNA binding proteins [26].

Disruption of activator function

Transcriptional repression can be achieved by processes that degrade, sequester, covalently modify, or alter the localization of activators in the nucleus (Figure 1-2A, insets 1-4) [26]. Additionally, disruption of activator function can occur through binding site competition, or by masking protein-protein interactions necessary for activation (Figure 1-2A, insets 5 and 6) [16]. For example, ubiquitination of p53 by Mdm2 can

promote protein turnover and nuclear export [27]. Similarly, deacetylation of E2F1 by the Rb complex can inhibit activator binding [28]. Another example of activator disruption is the sequestration of the MyoD activator by heterodimerization to the non-DNA-binding Id protein [26]. Other examples include masking of the Gal4 activation domain by the Gal80 repressor, binding site competition between the Engrailed repressor and the Fushi-tarazu activator, and blocking NFκB import into the nucleus by binding to the IκB repressor [29-31].

Direct repression of the basal transcriptional machinery

Some transcriptional repressors can disrupt transcription by direct interactions with the basal machinery in a promoter-specific manner (Figure 1-2B) [16]. Repressors can target the basal machinery by three known mechanisms. The first is through modification of the locally bound Pol II holoenzyme (Figure 1-2B, inset 1). For example, the Tup1-Ssn6 repressor complex has been suggested to mediate repression by interacting with the Srb10/11 cyclin/cyclin-dependent-kinase pair to misregulate Pol II CTD phosphorylation [32-34]. The second mechanism is by blocking TBP binding to the TATA box (Figure 1-2B, inset 2) [16]. The homeodomain-containing repressor Eve has been shown to interact with TBP and disrupt formation of the pre-initiation complex (PIC) [35]. Finally, repression can also involve inhibiting the protein-protein interactions between the general transcription factors (GTF) (Figure 1-2B, inset 3) [16]. While monomeric Kruppel can activate transcription, a Kruppel dimer can mediate repression by interacting with the TFIIEβ subunit and preventing PIC assembly [36].

Recruitment of chromatin modifying and remodeling complexes

Transcriptional regulation in eukaryotes takes place in a highly complex environment consisting of a dense DNA/protein matrix called chromatin. Typical eukaryotic genomes are in the order of 10^6-10^9 bp, which uncoiled would be 10^1-10^2 cm long. Since an eukaryotic cell is approximately 10-100 μm long, DNA must be tightly packaged in order to fit inside the nucleus. The basic packaging unit is called the nucleosome, which is a highly stable complex consisting of 147 bp of DNA wrapped 1.67 left-handed turns around an octamer of histone proteins (H2A, H2B, H3, and H4) [37]. The linker histone H1 loosely associates with the nucleosome and promotes chromatin compaction [38]. When viewed under an electron microscope, chromatin has the appearance of an 11-nm "beads on a string", with the distance between each nucleosome varying between 10-100 bp [38]. In turn, chromatin can be packaged in higher order secondary and tertiary structures [39, 40]. Early studies on chromatin structure classified chromatin as euchromatin or heterochromatin depending on the degree with which they stained with DNA-specific dyes like Giemsa [41]. The lighter staining euchromatic regions are characterized by being relatively gene rich and have a looser structure [40]. In contrast, heterochromatic regions stain darkly, contain fewer active genes, and are highly condensed [40]. Since then, studies have shown that euchromatin and heterochromatin differ at a nucleosomal and DNA level, with specific covalent modifications of both histones and DNA nucleotides associated with each region [16, 40].

Many repressors work by altering the chromatin structure in order to create a repressive state (Figure 1-2C). A common mechanism is through the recruitment of histone modifying enzymes such as histone deacetylases (HDACs) and histone

methyltransferases (HMTs) [26]. Active genes are generally associated with high levels of histone acetylation, possibly because acetylation of the N-terminal histone tails might decrease the stability of the nucleosome complex [42]. Alternatively, acetylation might target the binding of chromatin remodeling complexes such as SWI/SNF to create a looser chromatin structure [43]. Thus, recruitment of HDAC complexes like Rpd3, Hda1, and Sir2 can decrease histone acetylation and create a more compact chromatin structure [26]. Histone methylation is often associated with gene silencing [44]. Recruitment of HMTs such as Su(var)3-9 and SET1 can result in the specific methylation of the lysine residues H3 K9 and K27 respectively [26, 44]. Methylation of these residues can in turn promote the binding of repressive complexes like HP1 and Pc to silence gene activity [44]. Other known histone modifications include phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deimination, and proline isomerization [44]. Additionally, in many organisms DNA methylation plays an important role in gene silencing [26].

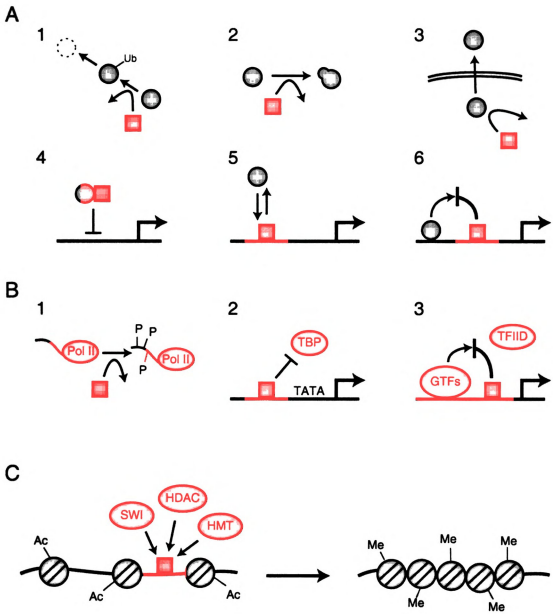

Figure 1-2. Mechanisms of transcriptional repression.

(A) Disruption of activator function can occur by degradation (1), covalent modification (2), translocation outside of the nucleus (3), sequestration (4), competition for binding sites (5), or blocking of activator interactions (6). **(B)** Alternatively, repressors can directly target the basal machinery by modifying the Pol II CTD (1), preventing TBP binding to the TATA box (2), or by blocking protein-protein interactions necessary to promote PIC assembly (3). **(C)** Additionally, repressors can recruit chromatin remodeling and modifying enzymes in order to create a repressive chromatin structure. Images in this dissertation are presented in color.

Hairy

The *hairy (h)* pair-rule gene encodes a 337-amino-acid transcriptional repressor that plays an important role in segmentation and sensory bristle formation in *Drosophila* [45-47]. Hairy is closely related to a family of proteins belonging to the Enhancer of split complex [E(spl)], which negatively regulate neurogenesis and are regulated by Notch signaling [48, 49]. Homologs for the Hairy/E(spl) (HES) have been found in both vertebrates and invertebrates, where they are involved in neurogenesis, neural cell fate, vascular development, mesoderm segmentation, and myogenesis [1, 50-53]. Hairy/E(spl) proteins are characterized by a conserved basic helix-loop-helix (bHLH) DNA-binding domain, an Orange repression domain, and a C-terminal WRPW motif that binds to the corepressor Groucho (Figure 1-3A) [54, 55]. Interestingly, studies of the mammalian *hairy/E(spl)* homolog HES6 suggests that the WRPW motif also acts as a protein degradation signal, promoting polyubiquitination and proteosomal degradation [56]. Hairy/E(spl) proteins are believed to bind to their targets as homodimers or heterodimers through interactions in the bHLH and Orange domains, with the Orange domain providing functional specificity [54, 57, 58]. However, yeast two-hybrid studies have shown that Hairy does not interact with any of the E(spl) proteins and binds presumably as a homodimer [57].

Initially Hairy is expressed in a seven stripe pattern in early *Drosophila* blastoderm embryos where it helps to establish the borders of alternate metameric segments in the fly by repressing the expression of the pair-rule genes *fushi-tarazu*, *runt*, and *oddskipped* (Figure 1-3B) [59, 60]. Later, in the larval stage, Hairy is involved in the

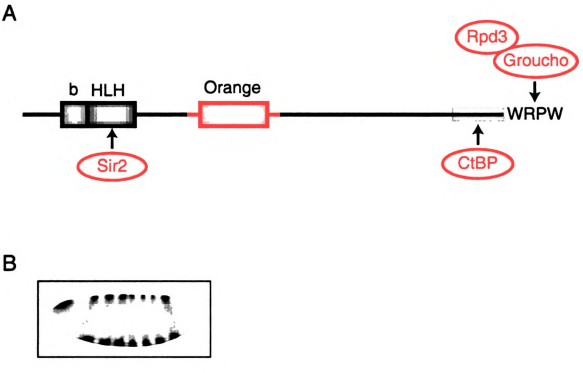

Figure 1-3. Conserved Hairy domains and expression pattern.

(A) Schematic diagram of Hairy showing the basic helix-loop-helix DNA-binding domain (bHLH), the Orange domain, and the C-terminal WRPW motif. Arrows point to the regions involved in interacting with the Sir2, CtBP, and Groucho corepressors. **(B)** *in situ* of *Hairy* mRNA expression pattern in early blastoderm embryos. Images in this dissertation are presented in color.

patterning of the sensory bristles in wing and leg imaginal discs through direct repression of the proneural gene *achaete* [61].

In contrast to short-range repressors, Hairy is able to silence gene activity even when bound 1 kb away from a heterologous enhancer [22]. However, in some cases Hairy can allow enhancer autonomy and act in a non-dominant fashion to locally repress enhancers [62]. The "hot chromatin model" has been proposed to explain the context dependency in the range of Hairy repression, this model postulates that Hairy binding to single sites might require the presence of nearby activators to "open" the chromatin. Alternatively multiple Hairy binding sites placed close together could enable cooperative protein-protein interactions, allowing Hairy to bind in the absence of activators [62].

Interestingly, while the long-range repression ability of Hairy has been studied only in the context of transgenic reporter constructs, the few promoter sequences that have been analyzed have Hairy binding sites placed very near activators or the transcriptional start site [22]. For example a single Hairy binding site at -302, 50 bp upstream of a cluster of 3 activator sites in the promoter region of *achaete* has been shown to be critical for repression [61]. Similarly, the mouse Hairy/E(spl) homolog HES-1 can negatively auto-regulate its own transcription by binding to 4 sites placed within 20-170 bp of the transcriptional start site [63]. Additionally, repression of *sex lethal* by the Hairy-related protein Deadpan occurs by direct binding to a region between 93-125 bp upstream of the start site [64]. Therefore, it is unclear whether the long-range repression activity of Hairy is actually required for silencing endogenous targets. Interestingly, the Dorsal morphogenic protein, which also interacts with Groucho and has long-range repression activity, can repress the *zerknult* gene by binding a silencer

element approximately 1 kbp upstream of the start site [65]. However, since it is not known what activates this gene at the early blastoderm stage, it is difficult to determine if Dorsal is behaving in a long-range fashion with respect to the activators.

Hairy/E(spl) repression mechanism

Basic HLH proteins bind as homo- or heterodimers to a consensus CANNTG sequence known as an E-box. Both Hairy and E(spl) proteins have been shown to bind to E-boxes. However, Hairy homodimers prefers to bind to an alternate CACGCG sequence [47, 61]. Interestingly, the E(spl) proteins and a heterodimer between the bHLH activators Daughterless and Lethal-of-scute can both bind to the same consensus E-box *in vitro*, suggesting that binding site competition could be a possible repression mechanism in some contexts [66].

A conserved characteristic of Hairy/E(spl) proteins is the ability to interact with the Groucho corepressor through a WRPW motif at the C-terminal end [55]. Multiple lines of evidence have shown that Groucho plays a key role in mediating Hairy repression [67, 68]. However, Groucho binding to Hairy is not the sole repression mechanism since Hairy can still repress *scute* even without the WRPW motif [54]. Indeed, the corepressors CtBP (C-terminal binding protein) and Sir2 (silent information regulator 2) have been shown to interact biochemically and genetically with Hairy [69-71]. CtBP, like Groucho is also a conserved transcriptional corepressor present in both vertebrates and invertebrates, but unlike Groucho, CtBP is thought to mediate short-range repression by binding to PXDLS-type motifs present on short-range repressors such as Knirps, Snail and Kruppel ([72-74]. The Hairy CtBP binding region is located only 9 amino acids from

the C-terminal WRPW and corresponds to a divergent PLSLV motif with weaker binding affinity than the consensus [69, 72]. The functional importance of CtBP-Hairy interaction is unknown, but genetic evidence suggests that CtBP can antagonize Hairy repression, as reduction in CtBP levels suppresses the Hairy mutant phenotype [69]. Additionally, Hairy mutations that enhance CtBP binding inhibit Hairy-mediated repression, while mutations that block CtBP interaction have the opposite effect [72]. Since the CtBP binding region is very close to the C-terminal WRPW, it has been suggested that CtBP and Groucho compete for binding to Hairy. However, both CtBP and Groucho can simultaneously bind a Hairy monomer *in vitro* [70, 72]. Intriguingly, CtBP seems to be required for proper expression of three putative Hairy target genes identified by DamID profiling, wherein tissue culture expression of a Hairy protein fused to the Dam methyltransferase leads to the methylation of the DNA surrounding the sites of Hairy recruitment [75]. However, it is unclear whether the CtBP requirement is direct or indirect.

Unlike the effect seen with CtBP, reduction in the maternal dSir2 levels causes an increase in the Hairy mutant phenotype, suggesting a positive contribution to Hairy repression [71]. In vitro binding assays have shown that dSir2 can bind to Hairy, and that the region of Hairy required corresponds to a RRAR motif present in the basic N-terminal part of the bHLH domain [71]. Additionally, damID profiling in Kc cells, wherein Hairy and dSir2 were fused to the DNA adenine methyltransferase (dam), revealed a 30% overlap between Hairy and dSir2 targets [75]. It seems likely that dSir2-Hairy interaction is important for Hairy repression, because a similar biochemical and functional interaction was shown for two human Hairy/E(spl) homologs hHES1 and

hHEY2, indicating that dSir2-Hairy interaction is evolutionary conserved [76]. Sir2 was first isolated as a gene involved in the repression of the yeast silent mating type locus; later it was shown to be involved in the establishment of silent heterochromatic states at yeast telomeres and rDNA [77, 78]. The molecular mechanism of Sir2 silencing seems to involve a NAD-dependent histone deacetylation activity of Sir2 and the spreading of a Sir2 complex over large distances [79]. This is accomplished by stepwise recruitment by Sir2 of Sir3 and Sir4 to nearby hypoacetylated histones, which in turn recruit more Sir2, generating a positive feedback loop [80]. This type of mechanism would be compatible with the long-range repression activity of Hairy.

The Groucho (Gro) corepressor is a widely conserved metazoan protein that is recruited by a large number of transcriptional repressors. Groucho does not have any intrinsic DNA-binding ability, instead relying on direct protein interactions to bind to the target gene. Two types of signature motifs have been found that interact with Groucho, the C-terminal WRPW and a FxIxxIL motif called the Engrailed homology-1 (eh1) [81, 82]. In humans, Groucho homologs are called Transducin-like-enhancer-of-split (TLE) and share with Groucho a glutamine-rich N-terminal tetramerization domain (Q-domain) and seven WD repeats, each 40 amino acids long (WD-40), placed in tandem near the C-terminus, which are thought to be involved in protein-protein interactions [83]. Gro/TLE proteins exert their influence on gene activity by multiple mechanisms. Like Sir2, Groucho-mediated repression appears to involve histone deacetylase activity, in this case through direct interactions with Rpd3 [84]. Additionally, Groucho binds hypoacetylated histone tails, suggesting a repression mechanism analogous to that of Sir2, wherein Groucho spreads along the chromatin by means of a positive feedback loop involving

Rpd3-mediated deacetylation of surrounding histones that could allow more Groucho binding [85]. Post-translational modifications of Groucho play a role in its repression activity, as phosphorylation of Groucho by dHIPK2 can relieve target gene repression [86]. Interestingly, phosphorylation of mammalian TLE by CK2 on Ser 239 can enhance its repression activity [87]. Genome-wide studies of Hairy, CtBP, Sir2, and Groucho binding have shown that Hairy colocalizes with different combinations of cofactors at multiple loci, suggesting that Hairy can repress by alternate mechanisms via context-dependent cofactor recruitment [75].

The ability of HLH proteins to form homo- or heterodimers raises the possibility of an additional repression mechanism involving heterodimerization between Hairy/E(spl) proteins and bHLH activators in order to titrate them off the DNA. An example of such a mechanism is the ability of the protein Extramacrochaetae (Emc) to block DNA binding of Daughterless (Da) and members of the Achaete-Scute Complex (AS-C) by heterodimerization through the mutually conserved HLH domain [88]. Some Hairy/E(spl) proteins can heterodimerize with Da/AS-C, however it is unlikely they work in this manner *in vivo* since target gene repression is abolished when the Hairy/E(spl) binding sites are mutated indicating that the repressor needs to be present at the gene for repression to occur [47, 61]. Furthermore, addition of Hairy/E(spl) proteins does not displace Da/AS-C from E-boxes *in vitro* [64, 88]. Interestingly, heterodimerization with activators does seem to play a role in repression for some vertebrate Hairy/E(spl) homologs. For example, HES5 can heterodimerize with the E47 HLH activator and prevent it from binding to DNA in *vitro* [89]. Similarly, the related Hey1 repressor can heterodimerize with MyoD to block myogenic terminal

differentiation, while Hey2 can block DNA binding by the ARNT/EPAS (HIF2) complex and suppress VEGF transcription [90, 91]. Likewise, HEY1, HEY2, and HES1 can interact with the PTF1-p48 activator to block exocrine cell differentiation in the developing pancreas [92].

Hairy/E(spl) functions in Drosophila

The high degree of functional conservation of Hairy/E(spl) repressors and bHLH activators that exist among different species has led some to speculate that both form part of a functional "cassette" involved in the establishment of cell fate decisions [93]. The best studied of these functions involves their role in prepatterning proneural clusters and subsequent cell fate determination within the cluster. A model system for this process is the formation of the *Drosophila* sensory bristles where precursor cells gives rise to shaft, socket, glial, and neuronal cells that make up part of the peripheral nervous system (PNS) [94]. Establishment of the proneural fate occurs through the activation of the proneural genes *achaete*, *scute*, *lethal of scute*, and *asense* belonging to the AS-C, as well as *daughterless* and *atonal* [95-97]. These activators belong to a class of bHLH proteins that typically function in opposition to the Hairy/E(spl) repressors to establish cell fate decisions. Along with *hairy/E(spl)*, they have coevolved to regulate cell differentiation in multiple tissues for both vertebrates and invertebrates [93]. In the case of PNS formation, repression of these genes by members of the Hairy/E(spl) family has been shown to prevent the formation of ectopic sensory organs [98-100] (Figure 1-4A). Initially, prepatterning of the PNS is brought about by direct repression of *achaete* by Hairy in cells outside of the proneural cluster, resulting in a complementary pattern between *hairy*

and *achaete* gene expression [47] (Figure 1-4B, C, and D). Cells inside the proneural cluster belong to a so-called equivalence group, where any one of them has the potential to become a precursor cell [101] (Figure 1-4D). Only one cell within the equivalence group will then go on to become a precursor cell, with the rest assuming an epidermal cell fate by means of lateral inhibition of proneural genes by E(spl) proteins in response to Delta/Notch signaling [95, 102] (Figure 1-4D).

In addition to regulating the formation of sensory bristles, *hairy* also plays a role in the differentiation of the eye. Like other arthropods, flies have compound eyes characterized by an array of repeating units called omatidia, each of which acts as an independent photoreceptor. In the Drosophila eye imaginal discs, development of each ommatidium starts with the differentiation of the R8 photoreceptor, which then recruits the surrounding cells to form the lens, cone, pigment cells, and visual receptor that make up the ommatidia [103]. The differentiation of the R8 cells is temporally and spatially controlled by a wave of differentiation called the morphogenetic furrow that sweeps from the posterior to the anterior of the eye imaginal disc [104]. *Hairy* has been shown to be part of a network of genes regulating the progression of the morphogenetic furrow, acting in conjunction with *emc* immediately ahead of the furrow to place undifferentiated cells in a pre-proneural state [104].

Apart from their role in neurogenesis, *Drosophila hairy/E(spl)* genes are also important in patterning of the somatic musculature. Analogously to PNS development, muscle formation involves the establishment of a pluripotent field of cells expressing high levels of the mesodermal specific *twist* activator [105, 106]. The regulatory mechanism by which *twist* gene expression is prepatterned in the mesoderm has not been

Figure 1-4. Hairy/E(spl) proteins pattern the PNS in *Drosophila.*

(A) Hairy mutants lead to formation of ectopic bristles which form part of the *Drosophila* peripheral nervous system [107]. **(B)** Hairy prepatterns the PNS by repressing the proneural gene *achaete*. **(C)** Schematic diagram of the *achaete* promoter showing binding sites for the Hairy repressor and the HLH activators AS-C and Daughterless. **(D)** Representation of the PNS patterning process by Hairy/E(spl) proteins. Hairy along with emc initial represses *achaete* expression in cells outside a proneural cluster. A single cell within the proneural cluster is selected to become the precursor cell. Selection occurs through the process of lateral inhibition via the Notch signaling pathway. The resulting precursor cell expresses genes that will lead to formation of the bristle sensory organ. Images in this dissertation are presented in color.

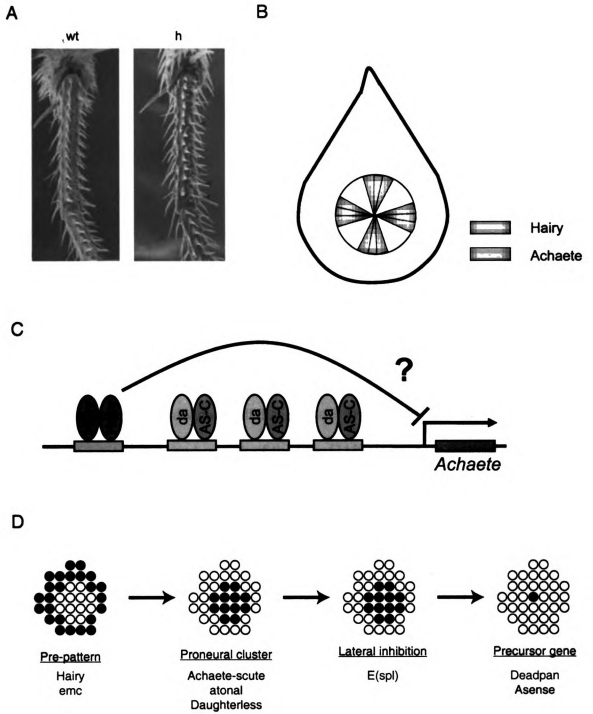

Figure 1-4. Hairy/E(spl) proteins pattern PNS formation in *Drosophila.*

fully elucidated, but evidence points to a role of Notch signaling both in the activation of *twist* inside the field as well as repression outside it through the actions of Supressor of Hairless [Su(H)] and members of the E(spl) class of repressors [108]. Once the pluripotent field has been formed, lateral inhibition of myogenic genes such as *lethal of scute* by E(spl) proteins in response to Notch activation, allows for a single precursor cell to be selected, which then divides into two myogenic founder cells that can in turn recruit surrounding cells to form the muscle [109, 110].

The *Drosophila* Malpighian tubes are part of the fly excretory system and form as invaginations between the hindgut and the foregut [111]. Following the same theme as before, development of the Malpighian tubes are characterized by the formation of an equivalence group of 6 to 8 cells that express the AS-C genes, followed by restriction of expression to a single non-dividing cell that migrates to the tip of the developing tube and regulates the invagination and growth of the tube [112]. It is likely that the selection of the tip cell involves E(spl) proteins, because mutations in the Notch pathway have shown misregulation of AS-C expression [112].

Deadpan is a transcriptional repressor involved in sex determination that is structurally related to the Hairy/E(spl) class, in that it contains a bHLH DNA-binding domain an Orange domain as well as a conserved WRPW motif [64, 100]. In flies, gender is determined by the ratio of X to autosomal chromosomes (X:A), where X:A = 1 leads to a female and a X:A = 0.5 leads to a male [64]. The genetic mechanism involves the transcriptional repression of the female-specific *sex-lethal* gene by Deadpan in response to the relative difference in X-linked sex determination genes or "numerator elements" such as *scute* (also known as *sis-B*), *sis-A*, *sis-C*, and *runt* [113].

Hairy has been found to regulate the formation of the *Drosophila* salivary gland secretory tubes, which are unbranched tubular structures that connect the salivary ducts with the larval mouth. Hairy works to suppress the formation of additional branches by repressing genes required for apical membrane growth such as *huckebein*, in cells outside the growth zone [114].

In summary, an important role of Hairy/E(spl) proteins in *Drosophila* is to determine the outcome of binary cell fate decisions (Figure 1-4D). The process involves establishment of an equivalence group of cells with the potential to assume a specific cell fate. Cells outside the equivalence group are prevented from assuming the same fate through repression of tissue-specific genes by Hairy. A single cell within the equivalence group then is selected to become the precursor cell of a particular cell type. Selection of the precursor cell is done by lateral inhibition of precursor-specific genes by E(spl) proteins as a response to Notch signaling.

Hairy/E(spl) functions in vertebrates

Vertebrate Hairy/E(spl) homologs (HES) are involved in many of the same processes seen in flies such as neurogenesis, myogenesis, and mesoderm segmentation, as well as roles in angiogenesis, and oncogenesis [1]. Thus, understanding the molecular basis by which these proteins work in flies would shed light on the transcription regulation of multiple developmental pathways in a wide range of organisms.

Paralleling the fly system, mammalian Hairy/E(spl) homologs HES1 and HES5 work to select neuronal precursors from a population of pluripotent cells in the developing nervous system [115]. Genetic interaction studies using mouse models

suggest that HES protiens function to suppress the expression of proneural genes such as *achaete-schute* homolog *MASH1* and *neurogenin1* (*ngn1*) in response to Notch signaling [115-117]. Similarly, the zebrafish E(spl) homolog HER4 can inhibit neurogenesis by repressing *ngn1*, while the *Xenopus* Enhancer-of-split related 1 (ESR1) protein can repress the proneural gene *Xath5* [118, 119]. In addition to their prepatterning role in the nervous system, HES1, HES5, and the *hairy/E(spl)* related hesr2 (also known as Hey2) have been implicated in determining the cell fate of neuronal precursors by dictating whether they will become neurons or glial cells [120-122]. In contrast to the other HES proteins, HES6 is expressed in neuronal precursors and promotes neuronal differentiation by forming a non-functional heterodimer with HES1 [123]. Along with their role in differentiating neurons, HES genes are also crucial for the maintenance of an undifferentiated neuronal stem cell population [124]. Overexpression of *HES1* or *HES5* in the developing mouse brain causes an overproliferation of undifferentiated neural stem cells, while knockdowns of *HES1*, *HES3*, and *HES5* cause premature neuronal differentiation and brain malformations [125, 126]. Recently, *HES1* has also been implicated in the formation of brain boundaries such as the zona limitans intrathalamica and the isthmus, which separate thalamus/prethalamus and midbrain/hindbrain respectively [127].

Another example of the importance of HES proteins in cell fate decisions can be seen in the development of the vertebrate blood circulatory system, where these proteins dictate whether cells will adopt a venous or arterial fate [128]. Evidence in zebrafish has shown that hypomorphic mutations or knockdowns of *hesr2* result in defects in dorsal aorta formation, which is correlated with a decrease in the arterial marker ephrinB2a and

an increase in the venal marker ephB4 [129]. The functional role of HES proteins in blood vessel formation is likely to be conserved in vertebrates because mutations of the mouse *hesr1* and *hesr2* likewise result in a loss of arterial identity [130, 131]. Analogous to other developmental processes, HES proteins act by repressing genes involved in vein formation such as *VEGF-A* and *VEGFR2* in response to Notch signaling [91, 132, 133]. It is interesting to note that the process of blood vessel formation in vertebrates is similar to the development of salivary ducts and Malpighian tubes in *Drosophila*, both of which are tubular structures. Since flies and other arthropods have an open circulatory system, one can speculate that evolution adapted a preexisting functional genetic cassette for generating tubular structures in order to develop blood vessels. The roles that HES proteins play in blood vessel formation suggest that they could be possible targets for cancer therapies seeking to block angiogenesis in developing tumors.

Recent studies have also implicated HES proteins in the development of proper heart morphology. Here, HES proteins are again involved in regulating cell fate decisions by specifying a chamber myocardium identity versus an atrioventricular canal (AVC) and inner curvature (IC) fate [134]. In chick embryos, the HES-related proteins Hey1 and Hey2 function to repress the gene *Bmp2* in cells destined to become part of the chamber myocardium [134]. *Bmp2* is exclusively expressed in the AVC/IC where it activates the gene *Tbx2* which in turn represses *Hey1* and *Hey2* expression; this feedback loop allowing a sharp demarcation between the AVC/IC and the chamber region [134]. Interestingly, only *Hey1* responds to Notch signaling, which makes it the direct effector in this context [134]. The regulatory mechanism for negatively regulating AVC/IC cell

fate seems to be evolutionary conserved, as similar mechanisms have been reported for both zebrafish and mouse [134, 135].

In addition to their role in patterning the heart AVC/IC regions, HES proteins have recently been discovered to be involved in valve and septum formation. Double mutants of mouse HES homologs *Hey1* and *HeyL* show severe malformations in the ventricular septum as well as in the pulmonary and atrioventricular valves. This was due to a failure to establish a mesenchymal fate by the epithelial cell precursors in the AVC. Establishment of the mesenchymal cell fate is likely to involve Notch signaling, since *Notch1*, *Notch2*, and the notch ligand *Jagged1* are coexpressed with *Hey1* and *HeyL* in the endocardium of the AVC. Additionally, a *Notch1* knockout abolishes the epithelial to mesenchymal transition [136]. It is interesting to note that Hey1 and Hey2 also seem to have a role in preventing calcification of the aortic valve (an important cause of heart disease in humans) by repressing the activity of the osteoblast specific Runx2 transcriptional activator and downstream target gene expression [137]. It is likely that a similar mechanism operates in humans because *Notch1* mutations have been shown to lead to aortic valve defects [137].

In the vertebrate embryo, patterning of the mesoderm occurs through a process of sequential segmentation from a terminal growth zone that leads to the development of groups of mesodermal cells called somites placed symmetrically along the neural tube [138]. In contrast, long germband insect segmentation like that of *Drosophila* occurs simultaneously by the parallel formation of seven stripes of gene expression from the pair-rule genes *hairy*, *even-skipped*, and *fushi-tarazu* in a syncytial embryo [45, 139]. Curiously, despite the difference in segmentation, Hairy functions in both insects and

vertebrates to prepattern the segmental body plan. Hairy regulates somitogenesis as part of a molecular oscillator termed the "segmentation clock", which generates waves of transcriptional activity that move from the posterior to the anterior in the presomitic mesoderm (PSM) [140]. For example, the chicken *hairy* homologs *hairy1*, *hairy2*, and *Hey2* are each expressed cyclically in the PSM, with *hairy1* and *Hey2* expressed in the posterior or caudal half of the developing somite, while *hairy2* is expressed in the anterior or rostral half [138, 141, 142]. Similarly, mouse *HES1* and *HES7* also show oscillatory expression patterns as well as the zebrafish homolog *her1* [141, 143, 144]. The molecular mechanism by which these oscillations occur has not been completely elucidated, but evidence points to the involvement of both extrinsic signaling via the Notch pathway, as well as cell-autonomous oscillations consisting of a negative feedback loop of HES proteins repressing their own transcription and subsequent proteosome-mediated degradation and reactivation [145-147].

As in *Drosophila*, HES proteins have also been implicated in skeletal muscle formation [90, 148, 149]. In the *Xenopus* embryo, overexpression of Hairy1 leads to repression of the myogenic inducing factor *MyoD*, while expression of a Hairy-VP16 chimera leads to *MyoD* upregulation, suggesting that Hairy1 binds directly to the *MyoD* promoter and represses it [148]. Furthermore, HES1 was shown to inhibit myogenesis in both rat and mouse derived cell lines [50, 150]. Also, another HES member CHF2 was shown to block *Myogenin* activation by MyoD in developing myoblasts by heterodimerization with the activator [90]. In contrast, mouse HES6 seems to be able to stimulate myogenesis by repressing transcription of the myogenic repressor *MyoR* [149]. Like other developmental processes where HES proteins are involved, it is likely that

Notch signaling is also active in myogenesis; overexpression of the Notch ligand *Dll1* in chick limb buds arrests myogenic differentiation and decreases *MyoD* expression [151]. Additionally, Notch signaling can inhibit myogenesis in both *Xenopus* embryos as well as in frog- or mouse-derived cell lines [152, 153]. The importance of HES proteins in myogenesis raises the possibility of a possible role in age or disease related muscle deterioration.

Another recently discovered role of HES proteins is in the developing thymus where they are required for proper T-cell differentiation and proliferation. In mice, *HES1* mutants mostly lack a thymus and show arrested T-cell differentiation at an early stage due to a lack of precursor cell proliferation [154]. It is not clear exactly how HES1 regulates T-cell differentiation, but it has been shown that HES1 directly repress the CD4 coreceptor by binding to a silencer element in the first intron of the gene [155, 156]. Proper transcriptional regulation of both *CD4* and the similar coreceptor *CD8* are crucial for T-cell differentiation and subsequent maturation [155]. Regulation of precursor proliferation has been suggested to occur through direct repression of the cyclin-dependent kinase inhibitor p27(Kip1) [157].

HES proteins have also been implicated in the development of the digestive system. For example, in the developing pancreas HES1 acts to maintain pancreatic stem cells by repressing *Ptf1a*, which promotes exocrine differentiation, as well as by repressing *Ngn3*, which promotes endocrine differentiation. *HES1* knockout mice show ectopic expression of *Ptf1a* and *Ngn3*, which leads to the formation of an extra pancreas [158, 159]. Similarly, HES1 is also important for the maintenance of the stem cell progenitors in the mouse intestinal epithelium by repressing MASH1 [160-162]. Loss of

HES1 activity causes the differentiation of intestinal stem cells into an endocrine cell fate, suggesting an involvement in specifying enterocyte versus non-enterocyte fates [160, 161, 163, 164]. Additionally, HES1 knockout mice show lack of bile duct formation in the liver, suggesting a role of HES1 in establishing a hepatocyte versus biliary epithelial cell fate [165].

In general HES proteins seem to have two crucial functions, the first one being to maintain stem cells in an undifferentiated proliferating state, while the second one involves regulating the outcome of binary cell fate decisions [124]. Disruption of HES activity in either of these functions has been shown to have both a positive and negative impact in oncogenesis. For example, some evidence suggests that *HES1* misregulation could be involved in the development of sympathetic neuroblastomas, as neuroblastoma cell lines show almost no *HES1* expression [166]. Furthermore, differentiating neuroblastomas show an upregulation of *HES1* and a downregulation of the proneural gene *HASH1* which is expressed in the sympathetic nervous system [166]. Similarly, studies have shown that activation of the Notch pathway and the downstream *HES1* effector can lead to inhibition of cellular proliferation in both gastrointestinal and lung carcinoma cells and direct repression of *HASH1* [167-170]. In contrast, inappropriate Notch signaling and HES activation is thought to promote the development of several T-cell leukemias by promoting undifferentiated precursor cell proliferation [171-174]. Likewise, inhibition of Notch signaling leads to differentiation of mice intestinal adenomas into post-mitotic goblet cells presumably by suppressing *HES1* expression [175].

Gal4

The yeast Gal4 protein has been used as a model for transcriptional activation for the past 20 years. In yeast, Gal4 functions to activate the expression of the *GAL* regulon when galactose is the sole carbon source [176]. Pioneering studies from several labs have led to a significant level of understanding of the molecular mechanisms by which Gal4 exerts its activity. In *Drosophila*, the Gal4/UAS system for the spatial and temporal control of gene activity has been a powerful tool for elucidating gene function [177].

Gal4 is an 881-amino-acid protein consisting of a Zn–Cys binuclear cluster-type DNA-binding domain, a linker domain, a dimerization domain and two acidic activation domains [176]. Gal4 binds as a homodimer to a 17-mer palindromic sequence 5' CGG AGG AC(T/A) GTC CTC CG 3' called the upstream activating sequence (UAS) [178]. DNA binding by Gal4 has been shown to be cooperative, with multiple UAS sites showing synergistic activation [179].

The mechanism of Gal4 activation seems to involve both the creation of an "open" chromatin state by recruitment of chromatin remodeling and histone modifying enzymes, as well as direct targeting of the Mediatior complex and Pol II holoenzyme to the promoter through interactions with its activation domain [180]. For example, in vitro studies have shown that Gal4 can interact with both TBP and TFIID [181, 182]. Similarly, biochemical and genetic interactions with Gal4 have been shown for Cdk8, Srb4, and Gal11 [183-185]. In addition, evidence has shown that Gal4 can also interact with the SAGA histone acetylase (HAT), as well as with the SWI/SNF chromatin remodeling complex [186, 187]. Interestingly, recent work has also implicated the proteosome subunits Sug1 and Sug2 as direct targets for Gal4 [188, 189].

The yeast Gal4 protein belongs to the class of "acidic" activators which includes the mammalian glucocorticoid receptor, c-myb, the viral VP16 protein, and the yeast Gcn4 [190-192]. Acidic activators are characterized by having short modular domains with a highly charged polar residues interspersed with hydrophobic groups [190]. The ubiquitous nature of these domains and the fact that they can function across species suggests an evolutionary conserved activation pathway. In *Drosophila*, the maternal morphogen *bicoid*, which specifies the anterior half of the embryo, also contains an acidic activation domain at the C-terminus [193]. Interestingly, expressing a chimeric protein containing the DNA-binding domain of Bicoid fused to a heterologous acidic activation domain can completely rescue the anterior pattern in a *bicoid* mutant embryo [194].

Gal4 transcriptional activation

Multiple lines of evidence have implicated SAGA as one of the main players in Gal4-mediated activation. Indeed, the importance of SAGA to gene activation has made it into one of the most well characterized HAT complexes. SAGA, which stands for Spt-Ada-Gcn5-acetyl transferase complex, is a 1.8 MDa complex containing the histone acetyltransferase Gcn5 as well as three other groups of proteins [195]. The first class consists of the Spt proteins Spt3, Spt7, Spt8, and Spt20/Ada5 that affect TBP function [195]. Spt3 and Spt8 have been shown to directly interact with TBP, suggesting a direct role in PIC assembly [195, 196]. The second class belongs to the Ada transcriptional adapter proteins Ada1, Ada2, Ada3. Previous work has shown that Ada2 and Ada3 are crucial for Gcn5 HAT activity *in vivo* and can also interact with TBP [197-199]. The

third class corresponds to a group of TBP-associated factors (TAFs) and Tra1, the latter which is shared with the NuA4 HAT complex [200, 201]. Recently, it was found that the deubiquitylase Ubp8 is also a part of the SAGA complex, this protein plays an important role in regulating histone H2B ubiquitylation [202].

Chromatin immunoprecipitation studies have shown that recruitment of SAGA is one of the earliest steps in an ordered process leading to Mediator recruitment and subsequent assembly of the pre-initiation complex (PIC) [203, 204]. In vivo imaging using fluorescence resonance energy transfer (FRET) has shown that Gal4 interacts directly with the Tra1 subunit of SAGA [204]. This interaction requires the presence of Spt20, a critical SAGA component required for complex integrity, suggesting that Tra1 needs to be in a complex to interact with Gal4 [204]. Interestingly, binding of Tra1 to Gal4 requires the presence of both the Gal4 DNA binding- and activation domains, suggesting that Gal4 needs to be bound to the UAS in order to interact with Tra1 [204]. In support of a direct Gal4-Tra1 interaction is the fact that SAGA recruitment by Gal4 can occur on a minimal UAS site and in the absence of a promoter [186]. Curiously, it seems that in some contexts Gcn5 is not required for PIC assembly, raising the possibility that SAGA actually acts as a scaffold for recruitment of the Mediator and Pol II holoenzyme [186]. It should be noted however that SAGA HAT activity seems to be important for the activation of some yeast genes such as *PHO5* and *PHO8* [205, 206]. In addition to its catalytic activity, Gcn5 also contains a bromodomain that interacts with acetylated lysine residues [207]. Importantly, functional homologs of the SAGA complex have been characterized in *Drosophila* as well as in other metazoans, suggesting a conserved mechanism of transcriptional activation [208].

In addition to SAGA, Gal4 activity has also been associated with recruitment of the SWI/SNF chromatin remodeling complex [187, 209]. SWI/SNF is an ATP-dependent chromatin remodeling complex that plays a key role in "opening" the chromatin to facilitate transcription. The exact mechanism of function is unclear, but recent *in vivo* evidence suggests that it acts by directly displacing the histone octamers from the DNA [210]. SWI/SNF consists of 11 subunits, of which the Swi2/Snf2 contains the ATP-dependent catalytic activity. In addition, Swi2/Snf2 also has a bromodomain, suggesting that histone acetylation can enhance SWI/SNF activity. Indeed, SAGA and SWI/SNF complexes seem to work in concert for the activation of many genes [43]. Furthermore, it has been shown that for some yeast genes like *HO*, SAGA-dependent histone acetylation is required prior to SWI/SNF recruitment [211]. Additional evidence of the functional importance of SWI/SNF in Gal4 activity comes from studies looking at the *GAL* genes. In the inactive state the promoter region of the *GAL* genes contains nucleosomes positioned over the TATA box and the transcriptional start site. Upon galactose induction these nucleosomes become disrupted in a Gal4-dependent manner, facilitating the recruitment of Pol II and PIC assembly [212]. It is likely that SWI/SNF is involved in this process, as chromatin immunoprecipitation studies have shown binding of Swi2/Snf2 to the *GAL1-10* promoter region upon Gal4 activation [213]. Like SAGA, functional homologs of the SWI/SNF complex have been found conserved from yeast to humans [214].

Apart from histone acetylation and nucleosome disruption, recent work has uncovered a role for histone ubiquitylation in Gal4-dependent activation. Transient monoubiquitylation of H2B K123 was shown to be required for maximum activation of

GAL1 [202]. H2B ubiquitylation and deubiquitylation seems to be a tightly coordinated process regulated by the Rad6 ubiquitin-conjugating enzyme and the deubiquitylation activity of the SAGA subunit Ubp8 [202, 215]. Targeting of Rad6 to the Gal1 promoter is dependent on the presence of both Gal4 and the E3 ligase Bre1 [215]. The kinetics of Rad6 binding follows very closely that of H2B ubiquitylation and seems to occur at a step immediately prior to SAGA recruitment [215]. After SAGA binding, monoubiquityl-H2B levels quickly go down as a consequence of Ubp8 activity [202]. Concomitant with the loss of monoubiquitylated H2B, Rad6 levels also drop, while SAGA remains bound to the gene [215]. The functional importance of H2B K123 ubiquitylation seems to lie in a "*trans*-tail" process whereby the ubiquitin moiety leads to the mono-, di-, and tri-methylation of H3 K4 and K79, which are dual purpose marks important for the activation and silencing of genes [202, 216, 217].

Mechanistic studies on the "*trans*-tail" process have uncovered close links between H2B ubiquitylation and transcriptional elongation. For example, Rad6 has been shown to interact genetically and biochemically with several elongation factors including Paf1 [218]. Furthermore, this interaction seems to be dependent on the presence of both Bre1 and the elongating form of Pol II, which is phosphorylated on Ser5 of the CTD [218]. Moreover, mutations that affect Pol II Ser5 phosphorylation or transcriptional elongation abolish H2B ubiquitylation and reduce Rad6 binding, with a resulting loss in H3 K4 and K79 methylation [218]. Like Paf1, the Set1 histone methyltransferase (HMT), which catalyze H3 K4 methylation, can also associate with the elongating Pol II [219]. This association is mediated by Paf1 and requires phosphorylation of Pol II Ser5 by the Kin28 kinase, which is part of the TFIIH complex [219]. It is likely that a similar

association occurs with the Dot1 HMT, which catalyzes H3 K79 methylation, as Paf1 knockouts result in complete loss of this modification [220]. Interestingly, it has been shown that H2B ubiquitylation is not required for monomethylation of H3 K4 and K79, rather it is needed for processive di- and trimethylation [221]. Lastly, recent studies suggests that phosphorylation of Ser120 of Rad6 by the Bur1/Bur2 cyclin-dependent protein kinase is necessary for full ubiquitylase activity [222]. Since Bur1 is required for efficient transcriptional elongation and is recruited to the elongating Pol II, it suggests a model where Rad6-Paf1 association allows Bur1 to activate Rad6 [223]. Thus, by associating with the elongating transcriptional complex, Rad6-mediated histone ubiquitylation could help target Set1 and Dot1 activity to the promoter and open reading frame (ORF). The resulting methylation marks could flag these regions for remodeling by the Isw1 and Chd1 chromatin remodelers, which have chromodomains that recognize H3 K4 methylation [224, 225].

Chapter II

Recruitment of Gal4 and SAGA is not affected by Hairy-mediated repression[1]

Introduction

Transcriptional repression plays central roles in developmental gene regulation, providing the temporal and spatial specificity required for complex expression patterns. In *Drosophila*, the Hairy transcriptional repressor directs patterning of segmental pair-rule stripes in the blastoderm embryo, and in later stages directs neuronal differentiation. [47, 61, 98, 226]. Hairy and related transcription factors belong to a conserved metazoan family of *H*airy *E*nhancer of *S*plit (HES) proteins involved in cell fate decisions in neurogenesis, vascular development, mesoderm segmentation, and myogenesis [1, 227]. Understanding the molecular basis by which these proteins exert their function will shed light on the transcriptional regulatory mechanism of multiple developmental processes.

In general, repressors can act by three basic mechanisms disruption of activator function, direct targeting of the basal machinery, or by chromatin remodeling [16]. Repressors typically use a combination of all three mechanisms in a wide array of alternate strategies. Thus, no common mechanism is shared by all repressors. However, the high degree of conservation between the different HES proteins suggests a similar mechanism of repression for this family.

The focus of this chapter is to investigate possible mechanisms by which Hairy could disrupt activator function. Disruption of activator function can occur by suppressing the formation of activator/DNA complexes or by blocking the protein-protein

[1] Parts of this chapter were excerpted from the paper titled "Spreading of corepressor linked to action of long-range repressor Hairy" by Carlos Martinez and David Arnosti, submitted to Molecular and Cellular Biology (2007)

interactions that activators make when bound to the DNA. Therefore, a key question to answer regarding Hairy repression is whether it is permissive to activator and coactivator binding. To this end, a highly defined embryo system has been set up so that a reporter construct behaves homogenously within the embryo in response to repressor and activator inputs. In order to achieve a tight ON/OFF response a *lacZ* reporter was placed under the control of a transcriptional switch. Activation of the reporter was accomplished by the ubiquitous expression of the yeast Gal4 protein under control of the constitutive *daughterless* promoter. Transcriptional repression was brought about by heatshock-induced expression of a LexA-Hairy fusion protein that lacks the Hairy bHLH DNA-binding domain as well as the Sir2 interaction domain (Figure 2-1A).

This approach provides several advantages. First, by studying Hairy-mediated repression in the context of a *Drosophila* embryo, tissue-specific effects can be minimized. Additionally, the highly defined nature of the system allows all possible genotypic combinations of activator and repressor transgenes to be analyzed. Thus, the individual contributions of the activator and repressor can be elucidated by studying the promoter state when just the activator or repressor is expressed. Moreover, the availability of high-grade LexA and Gal4 antibodies allows the tracking of both activator and repressor binding. Lastly, the activation mechanism of Gal4 has been extensively studied, simplifying the analyis of Hairy repression by providing an initial set of testable hypotheses [180]. Thus, the role of Hairy in counteracting each step in Gal4-mediated activation can be investigated.

Materials and Methods

Construction of a transcriptional switch system in *Drosophila* embryos. A Hairy-repressible transcriptional switch pC2L5U2L was constructed by placing binding sites for a LexA-Hairy fusion and the yeast Gal4 activator (UAS) into the P-element transformation vector pC4PLZ [228], 55 bp from the basal *transposase-lacZ* reporter and 350 bp from the divergently transcribed mini *white* reporter. Based on the architecture of previous modules shown to be effectively repressed in the embryo [229], the 212 bp regulatory region contains two LexA binding sites inserted 5' and two LexA sites 3' of five high affinity UAS sites derived from a modified *UAS-lacZ* plasmid [230]. Oligonucleotides bearing 2 LexA binding sites (DA-721/722) were cloned 5' of 5 tandem UAS sites into NotI/HinDIII sites and 3' into an SphI site (DA-641/642) in pBluescript SK+, and the regulatory switch was inserted as a NotI/SphI fragment into pC4PLZ at -55 and -350 bp from the *lacZ* and *white* transcriptional start sites respectively (5' GCG GCC GC **CTG TAT ATA TAT ACA G**CA TCT AGA ACC **TGT ATA TAT ATA CAG** AAG CTT GCC TGC AGG T [**CGG AGT ACT GTC CTC CG**A G]x5 CGG AGA CTC TAG CAT GG **CTG TAT ATA TAT ACA G**CA GGT ACC TG**C TGT ATA TAT ATA CAG** CAT GC 3', binding sites for LexA and Gal4 in bold, restriction sites underlined). This construct was introduced into flies by *P*-element mediated germline transformation as described [231] and inserted into the third chromosome. An auto-activating fly line was then generated by crossing and recombining the reporter line to a line containing a *daughterless* enhancer expressing Gal4 ubiquitously in the embryo (Bloomington Stock Center #5460). Finally, a heat shock-inducible LexA-Hairy fusion (*hsp70 LexA-Hairy*) was introduced by recombination onto the same chromosome. This

gene was created by joining a *Bam*HI/*Kpn*I fragment containing a Kozak sequence, initiator ATG, and coding sequence for the entire LexA protein (amino acid residues 1-202) in frame to a *Kpn*I/*Xba*I fragment containing the portion of Hairy C-terminal to the DNA-binding domain (residues 93-337), and introducing it into the *Bgl*II and *Xba*I sites of pCaSper-hs [232], (5' GGA TCC ACC AAA **ATG AAA...TGG CTG** GAA TTC CCG GGC CGG GGT ACC **GCA GCC...TGG TAG** TCT AGA 3'; coding sequences for LexA and Hairy in bold, restriction sites underlined, dots represent the rest of the coding sequence). The resulting line, containing all three transgenes on the third chromosome, behaves as a transcriptional switch in which the default state is ON before heat shock and OFF after induction of the LexA-Hairy repressor. The triple recombinant chromosome is carried over a TM3 Sb balancer chromosome and was found to be non-homozygosable. The *pKruppel-LexA-Hairy* plasmid was constructed by exchanging the Gal4 DNA-binding domain coding sequence from the pKreg vector [74] for LexA (residues 1-202) via restriction digest with *Bam*HI/*Kpn*I and ligation of a *Bam*HI/*Kpn*I LexA fragment PCR amplified from the pLexA vector (Clontech) using primer DA-645 (5' GCG GAT CCA CCA AAA TGA AAG CGT TAA CGG CCA GG 3') and DA-646 (5' CGG GGT ACC CCG GCC CGG GAA TTC CAG CCA GTC GC 3'. The reporter shown in figure 2-2 containing LexA binding sites 3' of the *rho* and *twi* enhancers was constructed by removing the Giant binding sites in the gt-55 vector [21] by SphI digestion and insertion of DA-641/2 (5' **CTG TAT ATA TAT ACA GC**A GGT ACC TG**C TGT ATA TAT ATA CAG** CCA TG 3') containing two LexA binding sites.

***In situ* hybridization and antibody staining of *Drosophila* embryos.** Embryos were fixed for *in situ* hybridization and stained using a digoxigenin-UTP labeled antisense RNA probe to *lacZ* as described [231]. Antibody staining for LexA-Hairy expression was done using a 1:2:1 mixture of three mouse monoclonal antibodies raised against LexA, YN-lexA-2-12 (2 μg/ml, 1:2100), YN-lexA-6-10 (4 μg/ml, 1:2023), YN-lexA-16-7 (4 μg/ml, 1:3950) obtained as a gift from Steve Triezenberg [233]. Quantitation of *lacZ* staining was done by scoring the stained embryos into unstained, lightly stained, and darkly stained from 10-13 random fields. Total embryos scored per slide varied between 135 and 457.

Formaldehyde crosslinking of embryos for chromatin immunoprecipitation. For chromatin immunoprecipitation assays, embryos (0.25-0.5 gm) were collected on 20% apple juice plates supplemented with yeast paste from 25-50 plastic laying bottles containing 300-500 flies each. Typically, fresh plates were placed in the laying bottles and embryos were subsequently collected for 3 hours and aged for 2 hours at room temperature. Low-level expression of the heat shock construct was observed in some cases, but not enough to repress *lacZ* expression (in 20 biological replicates, some LexA-Hairy signal was present prior to heat shock in half of the experiments, but most experiments showed 8-10 fold increase in signal after induction). Depending on the conditions of the experiment, embryos were then either immediately fixed, or heat shocked (typically for 20 min) by floating plates in a 38°C water bath and allowed to recover for a variable length of time at room temperature. Embryos were then collected in a nylon mesh and dechorionated with 100% bleach for 2.5 min and washed with

deionized water for 1 min For formaldehyde crosslinking, embryos were placed in a 50 ml Corning tube and fixed for 20 min with vigorous shaking in 10 ml 3% formaldehyde fixing buffer (50 mM HEPES pH 7.6, 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl) and 30 ml heptane. The formaldehyde was added from 37% stock (J.T. Baker # 2106-01) immediately before use. Embryos were then centrifuged at 3000 rpm (2000 x *g*) using a clinical Beckman centrifuge. Supernatant was removed and the crosslinking reaction was stopped by addition of 25 ml of crosslinking stop buffer (0.125 M glycine, 0.01% Triton X-100 in phosphate-buffered saline (1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4)) and shaking vigorously for 30 min After centrifugation as before and removal of the supernatant, embryos were either immediately processed for chromatin or flash frozen in liquid nitrogen and placed at -75°C.

Preparation of chromatin from whole embryos. 0.25-0.5 gm crosslinked embryos were washed in 10 ml embryo wash buffer (10 mM HEPES pH 7.6, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.02% sodium azide) for 10 min with vigorous agitation on a shaker, centrifuged at 3000 rpm (2000 x *g*) at 4°C and resuspended in 5 ml of sonication buffer (10 mM HEPES pH 7.6, 1 mM EDTA, 0.5 mM EGTA, 0.1% Triton X-100) and transferred to a 15 ml Corning tube. A proteinase inhibitor tablet (Roche, 11836153001) was added and the embryos were sonicated 30 sec (100% duty cycle) with 1 min cooling interval for a total of 12 times using a Branson sonicator S250 fitted with a tapered microtip 5mm in diameter. During each pulse the output was gradually increased from setting 1 to 7 to avoid foaming. Crude chromatin samples were aliquoted into microcentrifuge tubes, centrifuged at 14000 rpm (16000 x *g*) for 15 min at 4°C to remove

debris, and without disturbing the pellet, supernatant was transferred to a 15 ml Corning tube. An equal volume of 2xRIPA buffer (2% Triton X-100, 280 mM NaCl, 20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 0.2% SDS) was added. In experiments with multiple preparations, samples were visually inspected to match color and optical density, and in cases where they differed due to a different starting amount of embryos, total volume was adjusted by adding 1xRIPA, up to 20% of initial volume. Chromatin samples were then precleared by adding 10 μl/ml of a 50% slurry containing a an equal mixture of agarose beads coupled to protein A and protein G (Upstate, 16-125 and 16-266) that had been previously washed 3 times with 1xRIPA buffer. Chromatin samples were then aliquoted into microcentrifuge tubes in 1 ml fractions and either flash-frozen in liquid nitrogen and stored at –75°C or immediately immunoprecipitated. The DNA size distribution (measured after reversing cross-links) of typical chromatin preparations was less then 500-1000 bp.

Immunoprecipitation. Immunoprecipitations were carried out in microcentrifuge tubes by overnight incubation (12-16 hr) of 1 ml of precleared chromatin with the appropriate antibody at 4°C on a rotary mixer. At the same time, protein A and protein G agarose beads were mixed in equal proportions, washed 3 times with 1x RIPA buffer and then incubated overnight with 0.1 mg/ml BSA and 0.2 mg/ml salmon sperm DNA in 1xRIPA buffer. Before addition of mixed protein A/G agarose beads, chromatin/antibody reactions were centrifuged at 14000 rpm (16000 x *g*) for 15 min to remove insoluble material, 900 μl of the supernatant was transferred to a new tube, and 40 μl of 50% slurry of blocked protein A/G beads were added and incubated 4 h at 4°C on a rotary mixer.

Beads were centrifuged at 1000 rpm (80 x *g*) for 1 min and supernatant discarded, beads were washed with 0°C buffer 3 times with 1 ml low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8.0, 150 mM NaCl]), 3 times with 1 ml high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8.0, 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-Cl pH 8.0), and twice with Tris-EDTA (10 mM Tris pH 8.0, 1 mM EDTA), 3 min per wash. Chromatin was eluted by incubating the beads at room temperature in a rotary mixer with 250 μl freshly made elution buffer (1% SDS, 0.1 M monobasic $NaHCO_3$) pH 8.0, for 15 min Beads were centrifuged and the supernatant was transferred to a screw cap microfuge tube, a second elution was performed, and supernatants were combined giving a total of 500 µl. Twenty-five μl of 4 M NaCl was added to each tube and crosslinks were reversed by overnight incubation at 65°C. In parallel, 200 μl of input chromatin (20%) was mixed with 300 μl of elution buffer and incubated overnight at 65°C for input titration controls. To each tube, 10 μl 0.5 M EDTA pH 8.0, 20 μl 1 M Tris-Cl pH 6.5, 1 μl 10 mg/ml RNAse A, and 1 μl 20 mg/ml Proteinase K were added and incubated for 2 h at 42°C. DNA was extracted with 500 μl of phenol-chloroform, 400 μl of the aqueous phase was placed in a new microcentrifuge tube, and DNA precipitated by adding 1 μl (15 ug) Glycoblue pellet paint (Ambion), 44 μl 3 M NaOAc pH 5.2, and 444 μl isopropanol. Tubes were incubated (at room temperature to prevent SDS precipitation) for 1 h, centrifuged at 14000 rpm (16000 x *g*) for 15 min, and pellets were carefully washed with 0.5 ml 70% ethanol, dried at 65°C under vacuum for approximately 15-30 min and resuspended in 50 μl purified water (Millipore) and analyzed by PCR with the appropriate primers.

Antibodies for chromatin immunoprecipitation. The antibodies and amounts used for chromatin immunoprecipitation were as follows: non-specific mouse IgG (10 μg, Upstate), Rabbit anti-LexA (3 μg, Upstate), Rabbit anti-Gal4-TA (5 μg, Santa Cruz), Rabbit anti-dGCN5 (2 μl, gift from Jerry Workman), Rabbit anti-dAda3 (1 μl, gift from Jerry Workman), and Mouse monoclonal anti-Pol II-CTD 8WG16 (20 μl, Covance).

PCR analysis. Immunoprecipitated DNA was analyzed by PCR on a Robocycler Gradient 96 using Platinum hot-start polymerase (Invitrogen) to reduce primer-dimer formation. PCR analysis used 2 μl of immunoprecipitated or input DNA. The primer used to amplify the different regions of the reporter were *white* forward (DA-1027: 5'ATA CAG GCG GCC GCG GAT CTG AT 3'), *white* reverse (DA-1028: 5' AGA TAG CGG ACG CAG CGG CGA A 3'), promoter forward (DA-942: 5' ATC AGA TCC GCG GCC GCC TGT AT 3'), promoter reverse (DA-943: 5' CGT CCG CAC ACA ACC TTT CCT CTC 3'), +1kb forward (DA-865: 5' CGG GCG CTG GGT CGG TTA CG 3'), +1kb reverse (DA-873: 5' GGT GCC GCT GGC GAC CTG C 3'), +2kb forward (DA-948: 5' AAC CGT CAC GAG CAT CAT CC 3'), +2kb reverse (DA-949: 5' ATT CAT TCC CCA GCG ACC AG 3'), +4kb forward (DA 1012: 5' CGG TCG CTA CCA TTA CCA GT 3'), +4kb reverse (DA 1013: 5' ATT GTA ACA GTG GCC CGA AG 3'). Primers used to amplify intergenic regions were X intergenic forward and reverse (DA-954: 5' CAC AGT GGA CAC ATA CCA TAG 3' and DA-955: 5' CGG AAA ATA TCA GTG CGA AAG 3'), and chromosome 3 intergenic forward and reverse (DA-960: 5' GTT GAG AAT GTG AGA AAG CGG 3' and DA-961: 5' CGA AAA AGG AGA AGG CAC AAA G 3').

Densitometric analysis of gel images was performed using ImageJ software. Briefly, images were background subtracted using the rolling ball algorithm with a ball diameter of 50 pixels. A rectangular box was placed over the bands, and a plot profile was generated and saved as a tab formatted file. Microsoft Excel software was then used to analyze the plot profile, and a standard curve was generated using the area under the peaks of the input titration. Corresponding input percentage for each band was calculated by determining the area under the peak of each antibody, and interpolating against the standard curve. Typical experiments showed R^2 values between 0.98-0.99.

Gel images shown in figures 2-5 were background subtracted and the brightness and contrast adjusted so that the input titrations in experiments using multiple chromatin samples matched as closely as possible.

Results

Construction and characterization of a regulated Hairy repressible transgene. To characterize the activity of the Hairy repressor protein, we employed chromatin immunoprecipitation assays, which have been used extensively in yeast and cell-culture studies to identify protein complexes and chromatin modifications associated with genes during activation and repression. The advantage to these single-cell systems is that a relatively homogenous population of cells can be obtained, providing a "snapshot" of the promoters under different physiological states. Our objective was to study the activity of Hairy in the context of embryo development, but due to the spatially and temporally limited action of the endogenous Hairy protein, genes in a relatively small percentage of the total nuclei of the embryo would be repressed by Hairy at any point in time. Therefore, we engineered a gene regulatory system that allows us to place a constitutively expressed activator on the promoter, and induce the expression of a LexA-Hairy fusion protein in a facultative manner, converting the embryo from an "all on" to "all off" state (Figure 2-1B). The target gene contains five binding sites for the Gal4 activator, flanked by two pairs of LexA binding sites to accommodate the repressors; similar configurations had already been tested and shown to be repressed by endogenous Hairy protein [229].

To test the ability of LexA-Hairy to repress a Gal4-activated reporter in transgenic embryos, we expressed LexA-Hairy in a central stripe in embryos with a *lacZ* reporter regulated by the Gal4 activator or endogenous enhancers (Figure 2-2). Embryos expressing the LexA-Hairy gene driven by the *Kruppel* enhancer showed a wide swathe of understained nuclei in the central region, indicating that the repressor is functional (Figure2-2). We therefore constructed an inducible form of the LexA-Hairy transgene

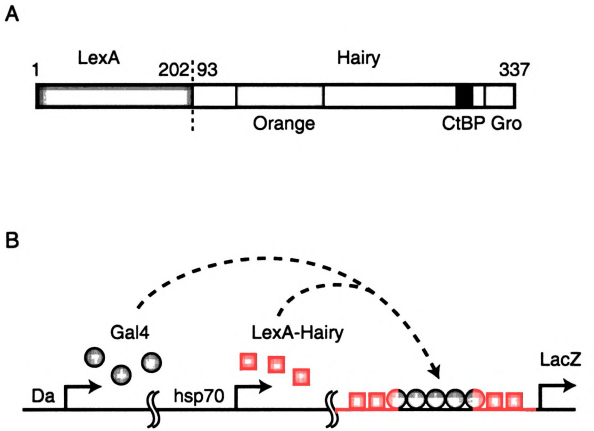

Figure 2-1. Hairy-regulated gene system in the *Drosophila* embryo.

(A) Schematic diagram of the LexA-Hairy fusion construct. The bacterial LexA protein was fused in frame to a Hairy fragment lacking the bHLH DNA-binding domain. The resulting construct contains the Orange, CtBP binding, and Groucho binding domains. **(B)** Three transgenes were combined onto a single chromosome to create a regulated on/off system; a *lacZ* reporter containing Gal4 and LexA sites, Gal4 activator driven by the *daughterless* (*da*) enhancer for broad expression in the embryo, and a heat-inducible LexA-Hairy construct. Gal4 and LexA-Hairy are represented by circles and squares respectively. Images in this dissertation are presented in color.

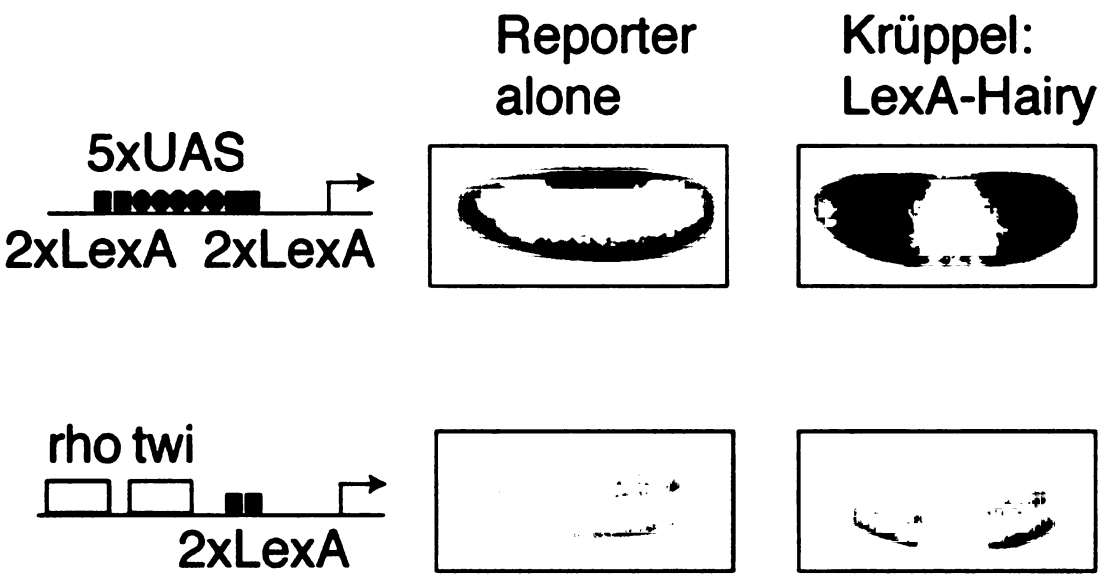

Figure 2-2. LexA-Hairy can repress *in vivo*.

In situ hybridization of *lacZ* mRNA expression of embryos carrying either a reporter, or a reporter and a LexA-Hairy transgene driven by a *Kruppel* enhancer. Upper, the *lacZ* reporter construct shown in Figure 2-1; below, a reporter activated by *rho* and *twi* enhancers, with LexA binding sites at promoter. In both cases, a central swathe of repression demonstrates effect of the LexA-Hairy chimera. Gal4 and LexA-Hairy are represented by circles and squares respectively. White rectangles represent enhancers. Embryos are shown anterior to the left, dorsal surface up. Images in this dissertation are presented in color.

under control of the *hsp70* promoter, allowing variable levels of induction by titration of heat shock conditions. As measured by in situ hybridization, *lacZ* transcript levels in the entire embryo dropped markedly after even a short (5 min) heat shock. Successively longer heat shocks resulted in complete loss of *lacZ* staining (Figure 2-3, 2-4). It should be noted however, that there was a great degree of heterogeneity in *lacZ* staining between embryos within each treatment class. Therefore, in order to get a more accurate reflection on the effect of LexA-Hairy repression, embryos within each group were scored with respect to *lacZ* staining as either "dark blue" (high *lacZ* expression), "light blue" (low *lacZ* expression), or "unstained" (no *lacZ* expression). Heat shock induction of LexA-Hairy resulted in a very obvious shift in *lacZ* staining with most embryos being classified as "unstained" by 30 and 60 min post-heat shock (Figure 2-5). In typical experiments, the percentage of embryos showing strong staining dropped from almost half to less than 1% one hour after induction of the repressor, indicating that the repression was effective in the vast majority of nuclei and embryos (Figure 2-5). Embryos aged for two hours after the heat shock induction showed restoration of *lacZ* expression, indicating that the repression is reversible (Figure 2-4). Heat shock treatment had no effect on embryos carrying the reporter gene and the activator in the absence of the LexA-Hairy repressor protein, indicating that heat shock itself does not interfere with transcription of this gene (Figure 2-3, lower panels).

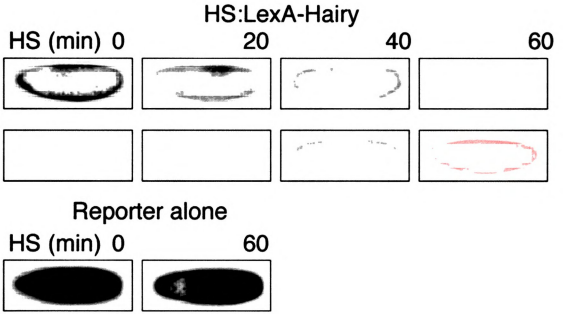

Figure 2-3. Expression of *lacZ* and LexA-Hairy in blastoderm embryos

Embryos containing transgenes were heat shocked for varying times and fixed for analysis of *lacZ* expression by *in situ* hybridization against the mRNA (top) or antibody staining of the LexA-Hairy protein (bottom). As LexA protein accumulates, *lacZ* mRNA decreases. Heat shock has no effect on *lacZ* in embryos lacking the LexA-Hairy protein (two lower embryos). Embryos are shown anterior to the left, dorsal surface up. Images in this dissertation are presented in color.

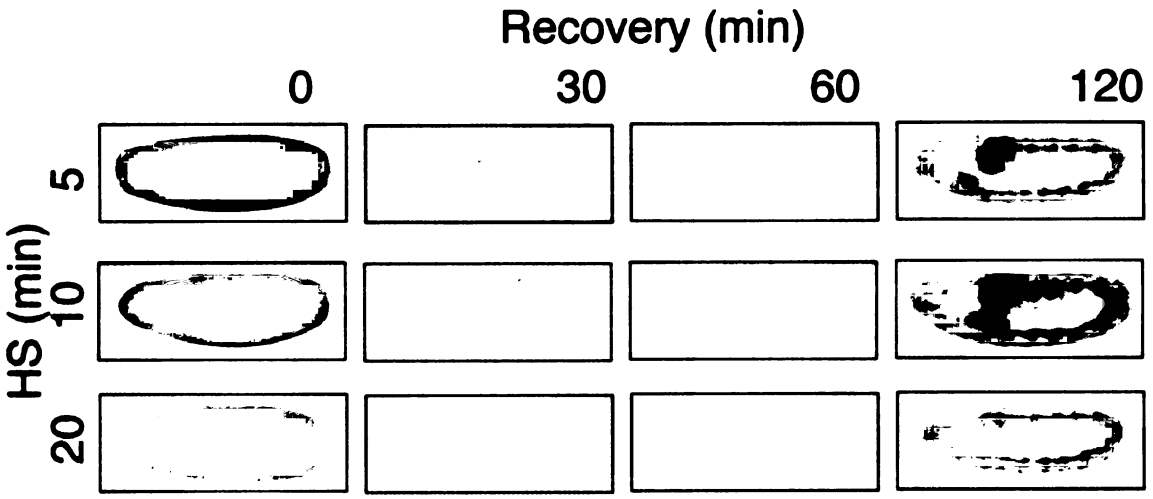

Figure 2-4. LexA-Hairy repression is reversible.

Repression and reactivation of transgenes in embryos after heat shocks of varying duration. Shown, *in situ* hybridization against *lacZ* mRNA. A 5 min induction of the LexA-Hairy protein is sufficient to cause significant loss of *lacZ* mRNA within 30 min RNA levels remain low at 60 min, and show recovery after 120 min (at this point, most embryos have aged to germband extended stage). Longer heat shocks (10 and 20 min) show similar recovery kinetics. Embryos are shown anterior to the left, dorsal surface up. Images in this dissertation are presented in color.

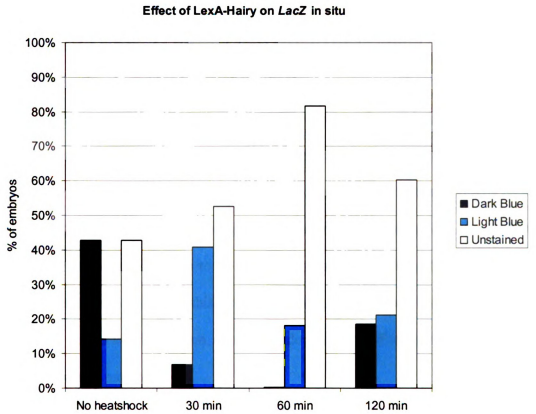

Figure 2-5. Quantitation of *lacZ* staining after heat shock induction of LexA-Hairy. 3-5 hour old embryos were either immediately stained or first heat shocked for 20 min at 37°C and then recovered at room temperature for 30, 60, and 120 min Embryos were then classified according to the level of *lacZ* staining as dark blue, light blue, or unstained. The percentage of dark blue embryos, corresponding to those with the highest amount of *lacZ*, drops dramatically after 30 and 60 min recovery. There is an apparent shift in the amount of *lacZ* staining from dark blue to light blue, and light blue to unstained, indicating that all embryos containing the reporter are becoming repressed. By 120 min *lacZ* expression has restarted with an increase in the percentage of stained embryos. Images in this dissertation are presented in color.

Co-occupancy of activator and repressor during gene repression. After establishing the efficacy of the system, large-scale collections were carried out, and 3-5 hour old embryos were treated with formaldehyde prior to chromatin preparation for immunoprecipitation reactions. We initially sought to detect the regulatory proteins that bound the promoter region. As expected, Gal4 protein was detected at the promoter only in lines carrying the *da*:Gal4 driver and the *lacZ* reporter gene (Figure 2-6A, top two panels). LexA-Hairy was readily detected at the promoter region, only in the presence of the *lexA-hairy* transgene. A very strong signal is detected at the promoter after the heat shock induction; a weak signal was also sometimes detected prior to heat shock, which is likely due to background expression of LexA-Hairy protein, although not at levels sufficient to inhibit transcription or to be detected by antibody staining (Figure 2-3). As a confirmation of the specificity of this interaction, neither Hairy nor Gal4 were found to associate with an intergenic region chosen randomly in another part of the genome (Figure 2-6A, lower panels).

Strikingly, the appearance of the LexA-Hairy repressor at the promoter did not preclude association of the Gal4 activator, indicating that the repressor appears not to rely on displacement of the activator for its activity (Figure 2-6A, fourth and fifth panels). A robust signal for the Gal4 protein was evident 30 minutes after induction of the repressor, at a time point when most *lacZ* mRNA has disappeared from the embryo (Figure 2-4, Figure 2-6A). The Gal4 signal does not represent the reassembly of active promoter complexes at this 30 min time point, because the embryos continue to show repression even at 60 minutes (Figure 2-4). The persisting Gal4 signal also does not represent material bound to a separate population of unrepressed promoters, because virtually all of

the embryos are uniformly repressed at this stage. The signal for Gal4 and LexA was strongly concentrated over the promoter region; considerably weaker signals were detected further 3' within the open reading frame (see Chapter3, Figure 3-1), possibly because of a higher order chromatin structure, or the presence of a small fraction of larger chromatin fragments >500 bp. In order to further confirm that Gal4 and LexA-Hairy can co-occupy the promoter, sequential ChIPs were carried out. The results show a specific signal for both LexA and Gal4 co-occupying the reporter during repression (Figure 2-6B). As negative controls, a LexA or Gal4 immunoprecipitation followed by IgG did not show any signal (Figure 2-6B). In contrast, LexA/LexA and Gal4/Gal4 sequential ChIPs showed a strong signal (Figure 2-6B).

Transcriptional co-activators remain associated with repressed promoter. The persistence of the activators at the promoter indicates that Hairy is not blocking their access, raising the question of why the promoter is not activated by Gal4 in this situation. We reasoned that repressor exclusion of co-activators might prevent Gal4 from having a stimulatory effect on the promoter. The Gal4 activation domain has been reported to recruit the SAGA co-activator complex [29, 234], therefore we carried out immunoprecipitations using antibodies against the Ada3 and Gcn5 subunits of SAGA (Figure 2-7). A promoter-localized signal for both of these proteins was detected in lines carrying the Gal4 activator (Figure 2-7, compare top two panels), consistent with the recruitment of the complex to the active promoter by Gal4. Binding of these coactivators was not detected at an intergenic locus chosen randomly in another part of the genome (Figure 2-7, lower panels). After induction of the Hairy repressor, the co-activators

Figure 2-6. LexA-Hairy repression does not prevent Gal4 binding.

(A) Promoter occupancy by Gal4 activator and LexA-Hairy repressor measured by chromatin immunoprecipitation. At top, no Gal4 or LexA-Hairy protein is detected with chromatin prepared from a line containing solely the *lacZ* reporter gene, as expected. The second panel contains a strong signal for the Gal4 protein, from chromatin containing the activated reporter. These embryos were heat shocked and recovered to parallel the treatment used for LexA-Hairy containing strains. Below, results from lines containing all three transgenes prior to and directly after induction of the LexA-Hairy repressor. Prior to induction, a signal for Gal4 is visible at the promoter, as well as a weak LexA-Hairy signal due to low level expression. After 20 min heat shock and 30 min recovery, a strong signal for LexA-Hairy is visible, in addition to the Gal4 signal. Both signals remain visible after 60 min recovery. LexA-Hairy signal drops by 120 min No signals are seen in the intergenic region on chromosome X. **(B)** Sequential chromatin immunoprecipitation of embryos expressing LexA-Hairy and Gal4 show that both proteins can co-occupy the promoter. Input titrations are shown for each chromatin preparation (2%, 1%, 0.5%, 0.25%, 0.125%, and 0.0625%). At left, results of quantitative PCR reactions, at right, heatshock and recovery conditions (heatshock done at 37 °C for 20 min, recovered at room temperature). Presence or absence of Gal4, and the heatshock-inducible LexA-Hairy transgene noted by +/-. Images in this dissertation are presented in color.

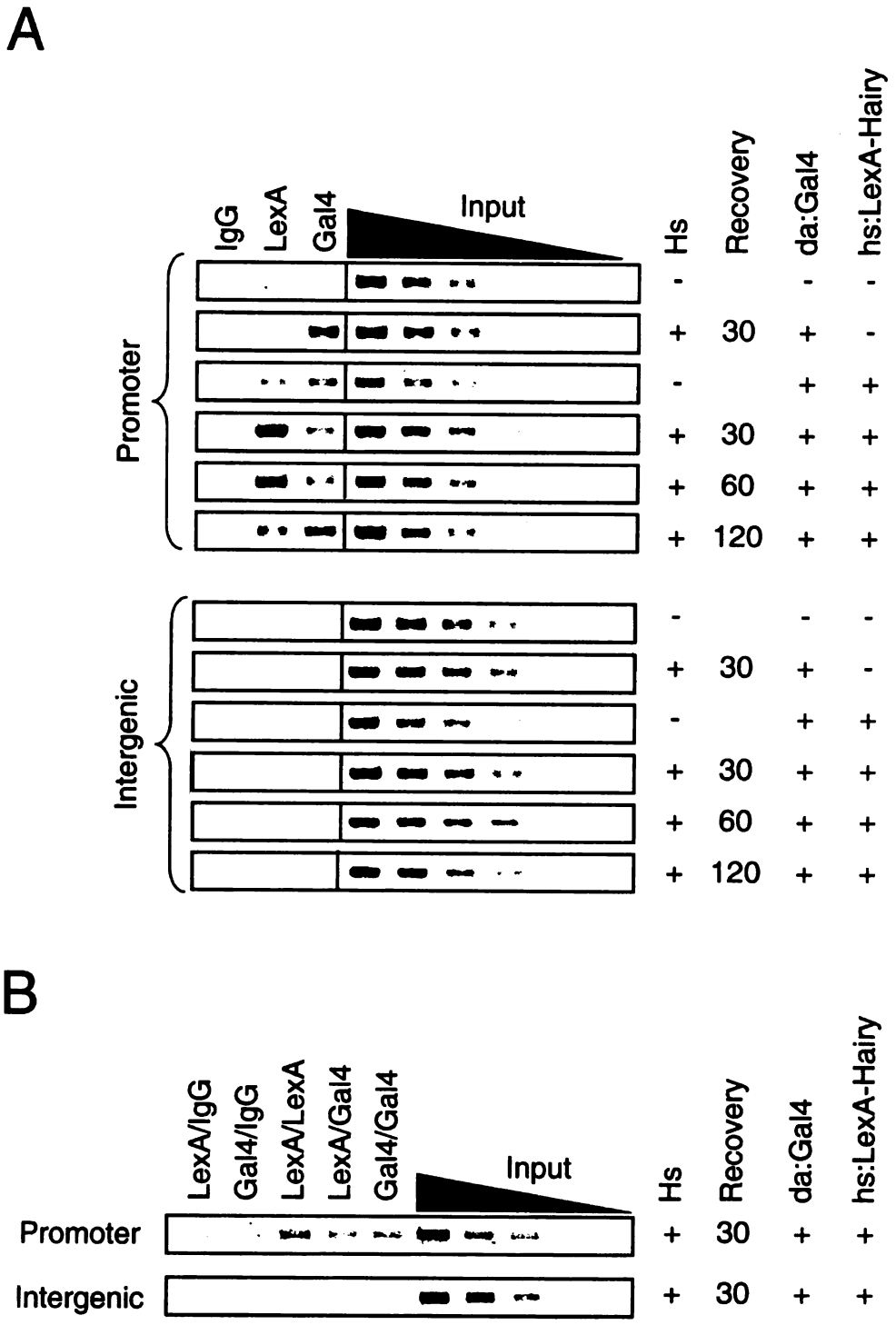

Figure 2-6. LexA-Hairy repression does not prevent Gal4 binding.

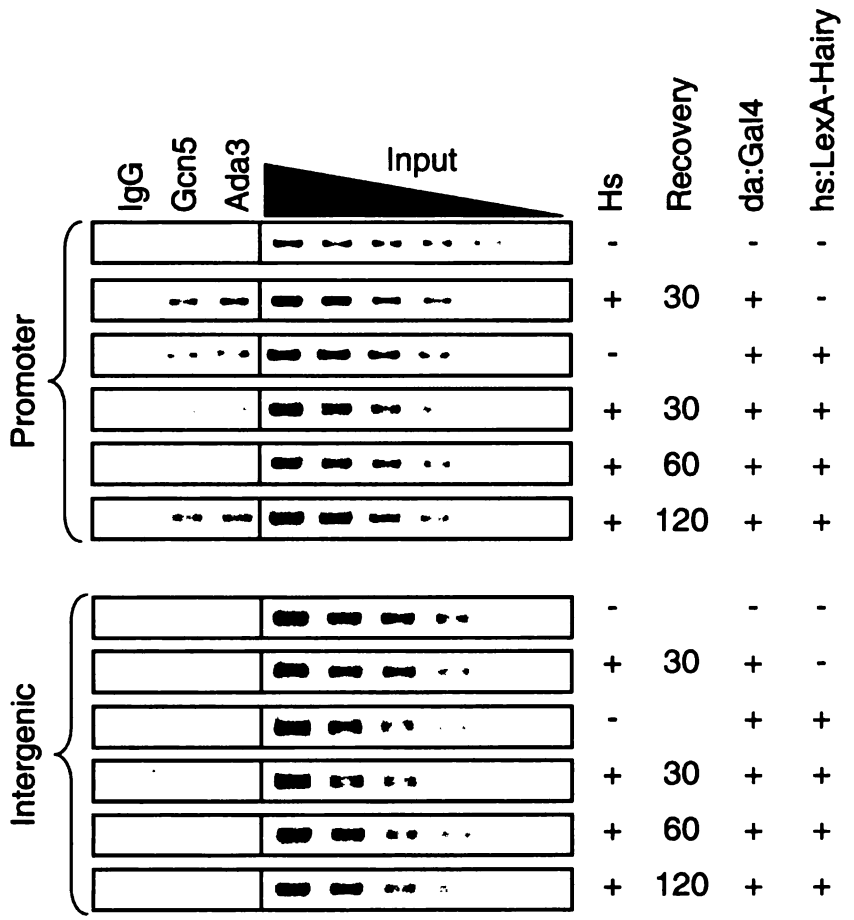

Figure 2-7. LexA-Hairy repression does not block SAGA recruitment by Gal4.

Promoter occupancy by SAGA coactivator constituents Gcn5 and Ada3 measured by chromatin immunoprecipitation. Protein occupancy of *lacZ* reporter promoter region in different states. The top panel shows no signal for these coactivators on the promoter lacking Gal4 activators, as expected. Both proteins can be detected on the promoter in a Gal4 activator containing strain, regardless of whether the embryos had been head shocked or not, and whether or not LexA-Hairy had been induced. No signals are seen in the intergenic region on chromosome X. Input titrations are shown for each chromatin preparation (2%, 1%, 0.5%, 0.25%, 0.125%, and 0.0625%). At left, results of quantitative PCR reactions, at right, heatshock and recovery conditions (heatshock done at 37 °C for 20 min, recovered at room temperature). Presence or absence of Gal4, and the heatshock-inducible LexA-Hairy transgene noted by +/-. Images in this dissertation are presented in color.

remained detectable at the promoter, at a point in time at which *lacZ* gene expression in the embryo has ceased (Figure 2-7, fourth and fifth panels). Thus, Hairy apparently does not exclude either activator or co-activator to effect repression of the promoter.

Discussion

Previously, repression had been thought of as being either passive or active. Passive repression was characterized as competition for binding sites between activators and repressors or the formation of an inactive repressor/activator complex [16]. For example the homeobox-containing repressor Engrailed competes for binding sites with the Fushi-tarazu activator [31]. Similarly, the bHLH protein Extramachrochaetae can bind to members of the Achaete-Scute complex and inhibit their binding to the DNA [88]. In contrast, active repression involves direct recruitment of the repressor to the locus where it can inhibit the basal machinery, block activator-promoter interactions, or change the chromatin structure. Evidence that Hairy is an "active" repressor comes from previous studies that have shown that Hairy can repress the proneural gene *achaete* by binding to a noncanonical E-box 50 bp upstream of three activator binding sites [47]. Furthermore, Hairy binding does not prevent activator binding *in vitro* [88]. Moreover, expression of a Hairy protein fused to a heterologous activating domain leads to transcriptional activation of putative Hairy targets, suggesting that it binds these targets directly [60]. In contrast, mutation of the Hairy binding site in the *achaete* promoter abolishes Hairy-mediated repression [61].

Our finding that activators and coactivators are still present under conditions when the gene is repressed by Hairy suggests that repression does not involve activator/coactivator displacement. The Gal4 activator might represent a particularly stably bound protein, as it does not show the high rate of exchange noted for other transcriptional activators [235]. Thus, it is possible that Hairy mediated repression does interfere with binding of some activators on endogenous loci. However, repression can be

quite effective even in the absence of activator displacement, perhaps by targeting the basal machinery, similar to Tup1-Mediator interactions seen in yeast [236-238]. One might suppose that the promoter proximal location of the repressor in our system might bias the system to such interactions, but this arrangement is physiologically relevant, as Hairy is found in such proximal locations on endogenous genes. In addition, the LexA-Hairy repressor is also active when bound at –2 kbp, indicating that promoter proximity is not required for activity (L. Li, unpublished observations). Interestingly, a recent chromatin immunoprecipitation survey of enhancers targeted by the Snail short-range repressor suggests that this repressor can be bound to inactive enhancers simultaneously with activators, raising the possibility that short-range repression might also involve direct interactions with the basal machinery [239].

Intriguingly, the results presented here point to a possible threshold effect for Hairy repression, where the levels of Hairy protein necessary to repress is directly proportional to the levels of activator present. Chromatin immunoprecipitation of LexA-Hairy and Gal4 in time course experiments showed a significant level of LexA-Hairy remaining two hours after induction at a point when transcription is restarting (Figure 2-4, 2-5, and 2-6). Similarly, before heat shock, there is a detectable LexA-Hairy signal presumably due to leaky expression of the repressor, yet the embryos show robust *lacZ* staining (Figure 2-4, 2-5, and 2-6). A possible explanation for these results is that it is not the total level of repressor bound but rather the ratio of activator/repressor binding to the gene that determines the transcriptional state. Thus, even in the presence of a significant amount of LexA-Hairy, repression is readily reversed by relatively higher levels of Gal4 activator.

The importance of such an effect to endogenous HES targets can be seen in the regulation of the sex determinant gene *sex-lethal*. Sex determination in *Drosophila* is determined by the ratio of X to autosomal chromosomes, where a ratio of 1 is female and 0.5 is male [64]. In males *sex-lethal* is repressed by the HES family member Deadpan by binding to a region between 93-125 bp upstream of the start site [64]. In contrast, *sex-lethal* becomes activated in females due to a higher transcription of X-linked activators (numerator elements) to *deadpan* (denominator element) [113]. Since *deadpan* is an autosomal gene, the concentration of the repressor will be the same in both females and males. Assuming that the fractional occupancy of Deadpan depends solely on the K_m and repressor concentration, then it should be similar in both males and females. Therefore, in order to maintain *sex-lethal* expression in females, activators must remain bound simultaneously with Deadpan and overcome its repression mechanism.

As mentioned in the first chapter, even in the absence of the Gcn5 histone acetyltransferase subunit, SAGA can still recruit the basal machinery through direct protein-protein interactions [186]. Since SAGA is not displaced upon LexA-Hairy repression, it is possible that Pol II is still being recruited to the promoter but is prevented from transcribing the gene. Preliminary chromatin immunoprecipitation experiments with Pol II antibodies showed a decrease in Pol II signal at the promoter after repression (Appendix B). Paradoxically, there was an increase in Pol II in the coding region, suggesting a paused complex (Appendix B).

Chapter III

Spreading of corepressor linked to action of long-range repressor Hairy[2]

Introduction

An important functional distinction between different repressors is their ability to interfere with proximally- or distally-located activators. In *Drosophila*, Hairy can inhibit the activity of activators located over 1 kbp away, leading to its characterization as a long-range repressor [22]. In contrast, short-range repressors are limited to interfering with activators bound within ~100 bp [18]. The limited range of short-range repressors appears to be well adapted to the architectures of the regulatory regions they control. In the *Drosophila* embryo, short-range repressors such as Knirps and Giant repress the modular enhancers controlling pair-rule genes such as *even-skipped* and *hairy*. The independent activity of such enhancers is guaranteed by the local action of the repressors; if enhancers are brought into artificially close proximity, or if binding sites for short range repressors are moved close to the transcriptional start site, unwanted cross-regulation can occur [19, 240].

In contrast, the molecular logic of *cis* regulatory elements controlled by long-range repressors is less obvious. At the *Drosophila achaete* gene, the long-range repressor protein Hairy binds at -300 bp, 50 bp 5' of a cluster of activator proteins, a position from which short-range repressors would also presumably work well [61]. Similarly, the Hairy homolog HES1 binds to its own promoter at four sites 20-170 bp from the transcriptional start site [63]. Dorsal protein-regulated ventral repression

[2] Parts of this chapter were excerpted from the paper titled "Spreading of corepressor linked to action of long-range repressor Hairy" by Carlos Martinez and David Arnosti, submitted to Molecular and Cellular Biology (2007)

elements (VRE) from *Drosophila zen*, *tld*, and *dpp* genes can similarly act over long distances, but at least in the case of the *zen* VRE, the activators bind immediately 5' of the repression element [241]. Thus, it is not clear if the long range of activity in these instances is essential to the normal regulatory function. Perhaps the strength of repression of Hairy is the most important feature, which is only incidentally associated with long-range effects.

Hairy/E(spl) proteins possess a conserved basic helix-loop-helix DNA-binding domain and effector domains that include motifs important for interaction with corepressors [1]. Hairy interacts physically and genetically with three corepressors: Groucho, the C-terminal binding protein (CtBP), and the Sir2 histone deacetylase [55, 69, 71]. The C-terminus of Hairy contains a WRPW motif that directly contacts the Groucho corepressor, and removal of the motif compromises the activity of Hairy. A motif adjacent to the Groucho interacting region binds to the CtBP corepressor. Hairy protein has been shown to possess CtBP-mediated repression activity in certain circumstances. However, CtBP has also been suggested to play an antagonistic role in repression by Hairy because binding of Groucho and CtBP might be mutually exclusive, and removal of the CtBP interacting motif has a less drastic effect on repression than removal of the Groucho motif [72]. The histone acetylase Sir2 interacts with the Hairy DNA-binding domain, and genetic interactions between *hairy* and Sir2 have been reported [71].

Whole genome mapping of binding sites for Hairy and cofactors indicates that at many loci, Hairy is not associated with all three cofactors. In fact, Hairy was rarely found to colocalize with regions bound by Groucho, while colocalization with CtBP was observed in a majority of cases [75]. These studies indicate that Hairy may associate with

specific cofactors in a context-dependent manner, perhaps invoking different modes of transcriptional regulation. A limitation of these studies is that the physical resolution is limited, so that it is not known whether Hairy and the corepressor proteins are in direct contact, or if in some cases other transcription factors might be recruiting these cofactors. In addition, it is not known for most loci whether the observed binding event is functional. Thus, while genetic and physical interactions hint at potential complexity, the activity of Hairy and its set of possible corepressors is not understood at a molecular level. To better understand molecular mechanisms of long-range repression, we have employed a novel approach to measure the activity of the Hairy repressor on a highly defined system in the *Drosophila* embryo.

Using transgenic lines containing a transcriptional switch that can be repressed uniformly in the embryo, we have analyzed the recruitment of the corepressors and histone modifications associated with Hairy repression. The results show that repression is associated with binding and spreading of the Groucho corepressor and the histone deacetylase Rpd3 throughout the coding region of a *lacZ* reporter. In addition, Hairy repression is associated with a marked decrease in histone acetylation levels and an increase in total histone occupancy. These results strongly support a model of Hairy repression involving the formation of a heterochromatin-like state.

Materials and Methods

Formaldehyde crosslinking of embryos for chromatin immunoprecipitation. For chromatin immunoprecipitation assays, embryos (0.25-0.5 gm) were collected from plastic laying bottles for 3 hours and aged for 2 hours at room temperature. Embryos were then either immediately fixed, or heat shocked (20 min.) by floating plates in a 38°C water bath and allowed to recover for a variable length of time at room temperature. Embryos were collected and dechorionated with bleach. For single crosslinking, embryos were fixed for 20 min with vigorous shaking in a 50 ml Corning tube in 10 ml 3% formaldehyde fixing buffer [50 mM HEPES pH 7.6, 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl, with formaldehyde (J.T. Baker # 2106-01) added immediately before use from 37% stock] and 30 ml heptane. Embryos were then centrifuged at 2000 x *g* in a clinical centrifuge, supernatant removed, and the crosslinking reaction stopped with 25 ml stop buffer [0.125 M glycine, 0.01% Triton X-100 in phosphate-buffered saline (1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4)], shaking vigorously for 30 min. Embryos were centrifuged as before and immediately processed for chromatin or flash frozen and stored at -75°C.

Double crosslinking of embryos for chromatin immunoprecipitation. Embryos were collected, heatshocked, and dechorionated as above, placed in a 50 ml Corning tube with 8 ml of phosphate-buffered saline. Two ml of a freshly prepared 25 mM dithiobis(succinimidyl propionate (DSP) crosslinking solution in DMSO was added to yield a final concentration of 5 mM DSP and 20% DMSO. Embryos were shaken

vigorously for 30 min., centrifuged at 2000 x *g*, supernatant removed, and crosslinked with formaldehyde as above.

Preparation of chromatin from whole embryos. 0.25-0.5 gm crosslinked embryos were washed in 10 ml embryo wash buffer (10 mM HEPES pH 7.6, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.02% sodium azide) for 10 min with vigorous agitation, centrifuged at 2000 x g at 4°C and resuspended in 5 ml of sonication buffer (10 mM HEPES pH 7.6, 1 mM EDTA, 0.5 mM EGTA, 0.1% Triton X-100) and transferred to a 15 ml Corning tube. A proteinase inhibitor tablet (Roche, 11836153001) was added and the embryos were sonicated 30 sec (100% duty cycle) with 1 min cooling interval 12 times using a Branson sonicator. With each pulse, the output was gradually increased from setting 1 to 7 to avoid foaming. The embryo lysate was aliquoted into microcentrifuge tubes, centrifuged at 16000 x g 15 min at 4°C, and without disturbing the pellet, supernatant was transferred to a 15 ml Corning tube. An equal volume of 2xRIPA buffer (2% Triton X-100, 280 mM NaCl, 20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 0.2% SDS) was added. For Groucho immunoprecipitation experiments from double crosslinked embryos the SDS was omitted. Chromatin was precleared by adding 10 μl/ml of a 50% slurry containing an equal mixture of agarose beads coupled to protein A and protein G (Upstate, 16-125, 16-266) previously washed 3 times with 1xRIPA buffer. Chromatin was then aliquoted into microcentrifuge tubes in 1 ml fractions and flash-frozen and stored at –75°C or immediately immunoprecipitated.

Immunoprecipitation. Immunoprecipitations were carried out by overnight incubation on a rotary mixer of 1 ml of precleared chromatin at 4°C with the antibody. At the same time, protein A and protein G agarose beads were mixed in equal proportions, washed 3 times with 1x RIPA buffer and then incubated overnight with 0.1 mg/ml BSA and 0.2 mg/ml salmon sperm DNA in 1xRIPA buffer. For Groucho immunoprecipitation experiments, 20 μg of Rabbit anti-Mouse IgG bridging antibody was added and the tube was incubated for an additional 1-2 hours. Before addition of mixed protein A/G agarose beads, chromatin/antibody reactions were centrifuged at 16000 x g for 15 min, 900 μl of the supernatant was transferred to a new tube, and 40 μl of 50% slurry of blocked protein A/G beads were added and incubated 4 h on a rotary mixer. Beads were centrifuged at 80 x g for 1 min, washed (0 °C) 3 times with 1 ml portions of low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8.0, 150 mM NaCl]), 3 times with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8.0, 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-Cl pH 8.0), and twice with Tris-EDTA (10 mM Tris pH 8.0, 1 mM EDTA. In Groucho immunoprecipitation experiments using double-crosslinked chromatin, 6 washes were performed with 1xRIPA lacking SDS (1% Triton X-100, 140 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). Chromatin was eluted at room temperature in a rotary mixer with 250 μl elution buffer (1% SDS, 0.1 M monobasic $NaHCO_3$, pH 8.0), for 15 min. Beads were centrifuged and the supernatant was transferred to a screw cap microfuge tube, a second elution was performed, supernatants combined, 25 μl of 4 M NaCl added, and crosslinks were reversed overnight at 65°C. In parallel, 200 μl of input chromatin (20%) was mixed with 300 μl elution buffer and

incubated overnight at 65°C for input titration controls. Then, 10 μl 0.5 M EDTA pH 8.0, 20 μl 1 M Tris-Cl pH 6.5, 1 μl 10 mg/ml RNAse A, and 1 μl 20 mg/ml Proteinase K were added and incubated for 2 h at 42°C. DNA was extracted with 500 μl of phenol-chloroform, 400 μl of the aqueous phase was placed in a new tube and DNA precipitated with 15 μg Glycoblue pellet paint (Ambion), 44 μl 3 M NaOAc pH 5.2, and 444 μl isopropanol. Tubes were incubated (at room temperature to prevent SDS precipitation) for 1 h, centrifuged at 16000 x g for 15 min, and pellets carefully washed with 0.5 ml 70% ethanol, dried at 65°C under vacuum for approximately 15-30 min and resuspended in 50 μl purified water for PCR analysis.

Using the simple formaldehyde crosslinking protocol, we sometimes obtained crosslinking of Groucho to the transcribed region but never to the promoter proximal sequences. This signal appears to be specific, because Groucho is never detected on other regions. Using the double crosslinking protocol, we found that Groucho, when detected, was also associated with the promoter, in addition to downstream regions. Even using this protocol, we were not always successful in detecting Groucho, consistent with the reported difficulty of detecting transcriptional cofactors indirectly bound to the DNA [242].

Antibodies for chromatin immunoprecipitation. We used the following antibodies: non-specific mouse IgG (10 μg, Upstate, cat # 12-371), rabbit anti-LexA (3 μg, Upstate, cat # 06-719), rabbit anti-Gal4-TA (5 μg, Santa Cruz, cat # sc-429), mouse monoclonal anti-Groucho (50, 100 μl, Iowa Hybridoma Bank), rabbit anti-HDAC1 (1-4 μl, Abcam, cat # ab1767), rabbit anti-H3 (1 μl, Abcam, cat # ab1791), rabbit monoclonal anti-

mono/di/trimethyl Histone H3 K4 (1 μl, Upstate, cat # 05-791), rabbit anti-acetyl Histone H4 (5 μl, Upstate, cat # 06-598), rabbit anti-dimethyl Histone H3 K27 (5 μl, Upstate, cat # 07-452), rabbit anti-acetyl Histone H3 (1 μl, Upstate, cat # 06-599), and rabbit anti-Mouse IgG (20 μg, Upstate, cat # 06-371).

PCR analysis. 2 μl samples of immunoprecipitated DNA was analyzed on a Robocycler Gradient 96 with Platinum hot-start polymerase (Invitrogen). Primers used were white forward (DA-1027: 5'ATA CAG GCG GCC GCG GAT CTG AT 3'), white reverse (DA-1028: 5' AGA TAG CGG ACG CAG CGG CGA A 3'), promoter forward (DA-942: 5' ATC AGA TCC GCG GCC GCC TGT AT 3'), promoter reverse (DA-943: 5' CGT CCG CAC ACA ACC TTT CCT CTC 3'), +1kb forward (DA-865: 5' CGG GCG CTG GGT CGG TTA CG 3'), +1kb reverse (DA-873: 5' GGT GCC GCT GGC GAC CTG C 3'), +2kb forward (DA-948: 5' AAC CGT CAC GAG CAT CAT CC 3'), +2kb reverse (DA-949: 5' ATT CAT TCC CCA GCG ACC AG 3'), +4kb forward (DA 1012: 5' CGG TCG CTA CCA TTA CCA GT 3'), +4kb reverse (DA 1013: 5' ATT GTA ACA GTG GCC CGA AG 3'). Primers used to amplify intergenic regions were X intergenic forward and reverse (DA-954: 5' CAC AGT GGA CAC ATA CCA TAG 3' and DA-955: 5' CGG AAA ATA TCA GTG CGA AAG 3'), and chromosome 3 intergenic forward and reverse (DA-960: 5' GTT GAG AAT GTG AGA AAG CGG 3' and DA-961: 5' CGA AAA AGG AGA AGG CAC AAA G 3').

Densitometric analysis of gel images was performed using ImageJ software. Gel images shown in figures 2-5 were background subtracted and the brightness and contrast

adjusted so that the input titrations in experiments using multiple chromatin samples matched as closely as possible.

Results

Association of Groucho and Rpd3 co-repressors with wide tracts of repressed gene. Using the defined embryo system (described in Chapter 2), we tested whether the corepressor Groucho was recruited to the *lacZ* reporter during repression (Figure 3-1). Using formaldehyde crosslinking techniques, Groucho was detected after induction of the Hairy repressor. No signal was detected at the promoter. However, strong signals were evident at regions 3' of the promoter at +1 kbp and +2 kbp, and less signal was detected at +4 kbp (Figure 3-1B, lanes 6 and 7). Groucho was not found to crosslink to an intergenic region, indicating that the signal is specific to the Hairy-repressed gene (Figure 3-1B). A similar pattern of crosslinking was obtained with the Rpd3 histone deacetylase, with strongest signals observed at +1 and +2 kbp (Figure 3-1B, lanes 3-5). This deacetylase has been identified as a Groucho-interacting protein in biochemical assays [84].

Detection of Groucho using this crosslinking protocol was variable, therefore we tested a double crosslinking procedure recently described for crosslinking of cofactors in yeast, which involves a two-step treatment employing the bifunctional crosslinker DSP first followed by formaldehyde [243]. Using this procedure we were again able to detect Groucho on downstream regions of the *lacZ* reporter gene. Under these crosslinking conditions, a strong signal was also detected over the promoter region (Figure 3-1C, lane 5). Again, no Groucho was found on intergenic regions, indicating the specificity of the signal (Figure 3-1C). The signal seen for the LexA-Hairy protein in this experiment is also detected more weakly at regions within the transcribed region, suggesting that there are protein contacts with sites distal to the binding sites within the promoter (Figure 3-

1C, lane 3). The extended signal is not simply the result of very long chromatin fragments, because the average chromatin size in this experiment is less than 500 bp, and the Groucho signal seen in Figure 3-1B is centered over the transcribed region, not the promoter. These widespread contacts of Groucho with the transcribed region of the gene are consistent with a model proposed by Courey, in which multimerization of protein through the N-terminal domain forms an extended "spread" conformation that permits the protein to influence multiple regions of the DNA, consistent with the long-range activity of Hairy [244].

We tested whether Hairy recruits Groucho to upstream regions, similar to the pattern seen on the transcribed locus. More distal regions could not be specifically sampled because of the presence of three copies of the *white* gene on the different *P*-element vectors in these strains. However, PCR primers specific to the promoter and the upstream mini *white* transgene were used to amplify proximal regions 5' of the promoter. These experiments showed recruitment of Groucho in chromatin prepared using the double crosslinking protocol (Figure 3-1C), suggesting that Groucho may be bidirectionally disposed around the LexA-Hairy binding sites.

We sought to detect the CtBP and Sir2 corepressors at this gene, but we did not reliably detect signals above background. Sir2 interaction with Hairy has been mapped to the DNA-binding domain, which is absent in this LexA-Hairy chimeric protein, thus it is not surprising that no signal was detected for this protein [71]. The negative result regarding CtBP is not very conclusive, because this particular antibody may not be suitable for immunoprecipitations. Nonetheless, repressors such as Hairy can utilize a

Figure 3-1. LexA-Hairy repression results in recruitment of Groucho and Rpd3 to the entire gene locus.

Occupancy of promoter and transcribed regions of a *lacZ* transgene by Groucho and Rpd3 corepressors during repression by LexA-Hairy. **(A)** Schematic diagram of LexA-Hairy regulated gene, showing portions amplified. **(B)** PCR analysis of formaldehyde-crosslinked chromatin immunoprecipitated from embryos that were induced for expression of the LexA-Hairy repressor for 20 min. and allowed to recover for 30 min. As expected, no signal was seen for the nonspecific IgG precipitation. A strong promoter-localized signal was detected for LexA-Hairy, with weaker signal on distal regions of the gene. Signals for the Rpd3 histone deacetylase (1, 2, and 4 μl of Rpd3 antibody shown) and the Groucho corepressor (50 and 100 μl of Groucho antibody shown) were detected at +1 kbp and +2 kbp, with a weak signal for Groucho at +4 kbp No crosslinking was detected for an intergenic region on chromosome 3. Input titration shown at right (2%, 1%, 0.5%, 0.25%, 0.125%). **(C)** PCR analysis of immunoprecipitated DSP+formaldehyde crosslinked chromatin. LexA and Gal4 are detected at the promoter, with weaker signals in 3' regions. In contrast to results in (B), a strong Groucho signal is detected at the start of the white gene (-350 bp), promoter, and throughout the transcribed region. No crosslinking was detected for an intergenic region on chromosome X. Groucho was not detected at the transgene locus in chromatin samples derived from single or double crosslinked embryos that were not heat shock induced for LexA-Hairy, or were heat shocked but lacked the *lexA-hairy* gene (not shown). Input titration shown at right (10%, 2%, 1%, 0.5%, 0.25%). Images in this dissertation are presented in color.

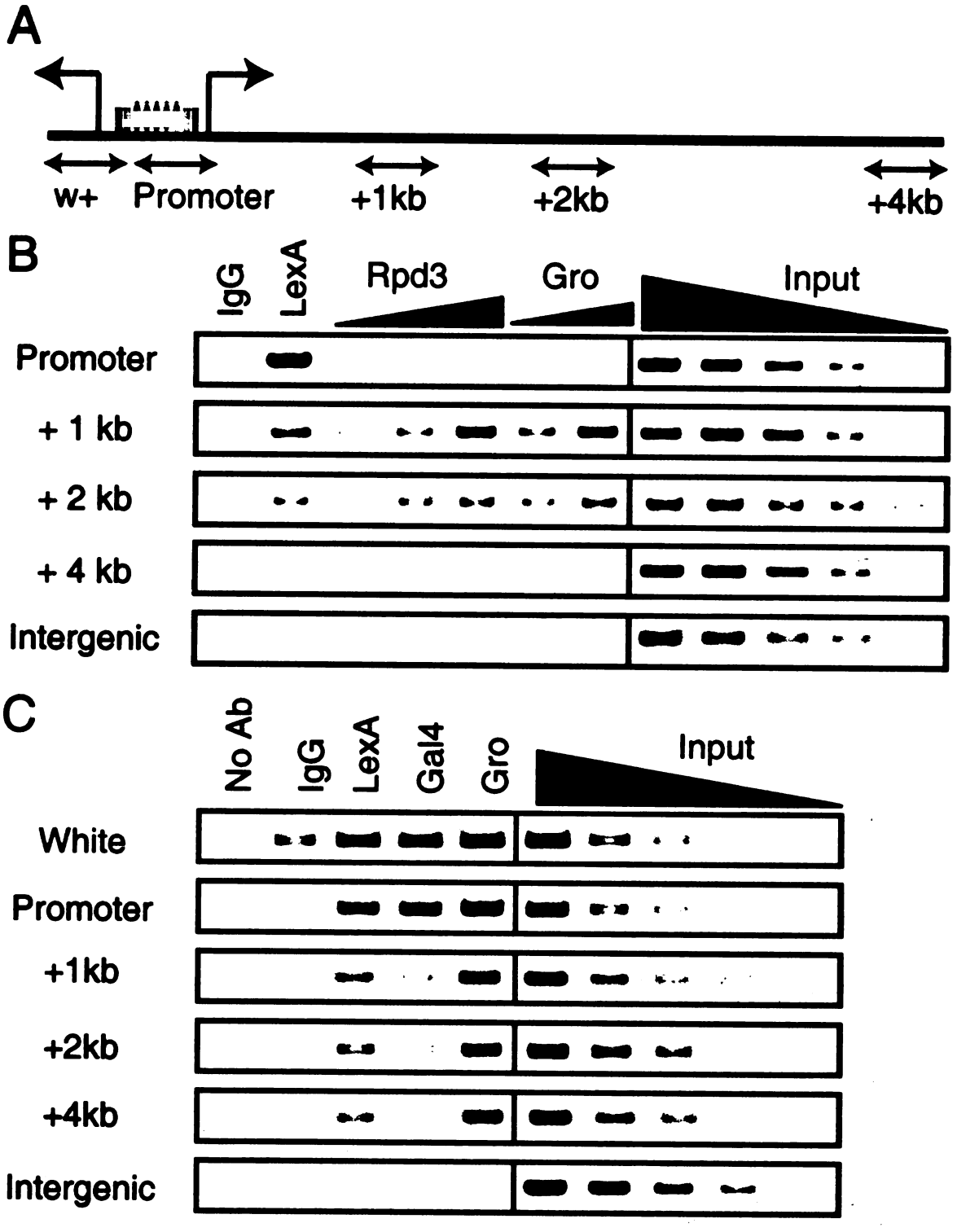

Figure 3-1. LexA-Hairy repression results in recruitment of Groucho and Rpd3 to the entire gene locus.

subset of corepressors for regulation of individual target promoters, and perhaps CtBP is not recruited to this gene [75].

Chromatin modification associated with activation and repression of a target gene. In light of the numerous connections between chromatin modifications and gene regulation, and the association of the deacetylase Rpd3 with the reporter, we tested the effects of gene repression on histones and chromatin modifications in the promoter region (Figure 3-2). Chromatin was prepared from embryos carrying only the reporter gene (unactivated state), embryos with both reporter and activator (activated state), embryos with reporter, activator and repressor, induced and aged for 30 to 60 min (repressed state), and 120 min (reactivated state). Recruitment of Gal4 to the reporter gene was associated with an overall reduction of levels of histones, measured by anti-H3 antibody (Figure 3-2B, third column). The levels of histone H3 and H4 acetylation, relative to total histone H3, were elevated, consistent with the appearance of SAGA subunits at the promoter (Figure 2-7, Chapter 2). Also associated with activation of the gene is a relative decrease in histone H3 K27 methylation (Figure 3-2B, fourth column). The induction of LexA-Hairy and repression of the gene is associated with an increase in overall H3 levels, and a relative decrease in H3 and H4 acetylation levels, consistent with the recruitment of histone deacetylases to the gene (Figure 3-2B, first and second columns). By these measures, the effect of the repressor is the opposite of the activator. However, the drop in relative H3 K27 methylation levels is not reversed by recruitment of the repressor, indicating an association of this modification with activation, but not repression, pathways. None of these effects were noted on a distal intergenic locus,

Figure 3-2. LexA-Hairy repression results in histone deacetylation and higher histone occupancy at the promoter.

(A) Chromatin remodeling, assayed by chromatin immunoprecipitation. Total histone H3 levels, acetylation, and methylation were assayed in the promoter region of the reporter gene in unactivated, activated, and repressed states, as well as with an unactivated reporter gene with repressor bound. The top two panels demonstrate the effect of Gal4 activators on the promoter; in the presence of Gal4 (second panel), overall histone H3 levels drop, as do H3 K27 methylation levels, while relative acetylated histone H4 and H3 levels increase. In the presence of the LexA-Hairy repressor (compare second and third panels), 30 min after induction, total H3 levels increase modestly and relative H3 and H4 acetylation levels drop. In this experiment, H4 acetylation levels were more affected. Relative levels of H3 K27 methylation remained low in the repressed state. The binding of repressor to the nonactivated gene (compare first and fourth panels) did not affect total histone H3 occupancy or H3 K27 methylation, and had only modest effects on relative acetylation. Below, relative levels of histone modifications at an intergenic locus on chromosome X are unchanged by the treatments. Immunoprecipitations were carried out with antibodies that recognize total histone H3, K9/14 acetylation of histone H3, K5/8/12/16 acetylation of histone H4, or K27 dimethylation of histone H3. Input titration shown at right (10%, 2%, 1%, 0.5%, 0.25%, 0.125%). **(B)** Quantitation of chromatin modifications at the promoter during activation, repression, and reactivation. Levels of acetylation and methylation were determined using densitometry analysis. Signals were normalized to the unactivated state. Shown are the average values and standard deviation of at least 5 biological replicates for the activated and repressed states. The average value of two biological replicates is shown for the reactivated state. Signals for AcH3, AcH4, and H3 K27 methylation are divided by the signal value of H3 to take into account differences in total histone levels. At left, results of quantitative PCR reactions, at right, heatshock and recovery conditions (heatshock done at 37 °C for 20 min, recovered at room temperature). Presence or absence of Gal4, and the heatshock-inducible LexA-Hairy transgene noted by +/-. Images in this dissertation are presented in color.

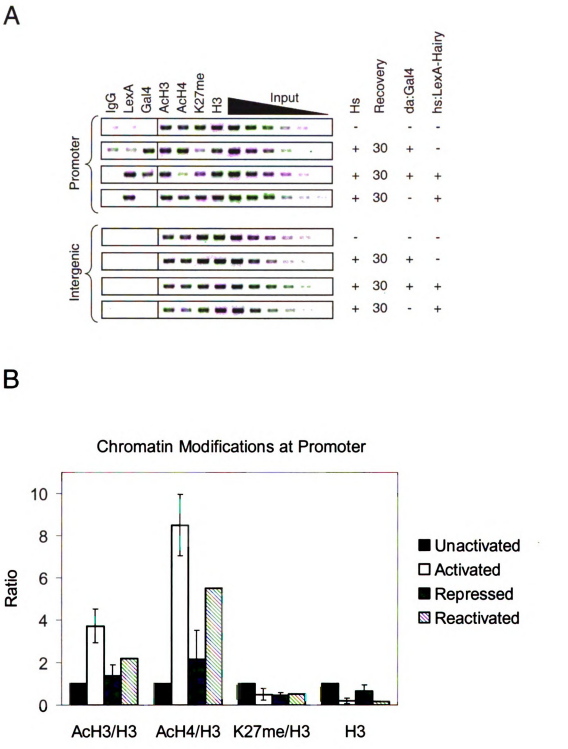

Figure 3-2. LexA-Hairy repression results in histone deacetylation and higher histone occupancy at the promoter.

indicating that the modifications were specific to the reporter gene (Figure 3-2A, lower panels). In addition, heat shock alone did not visibly alter chromatin signals, indicating that the heat induction regimen alone was not accountable for these changes (Figure 3-2A, lower panels). During reactivation, the changes in acetylation levels of H3 and H4, as well as total histone H3 were reversed back near the activated state (Figure 3-2B). No change in H3 K27 methylation levels was observed (Figure 3-2B).

Previous studies of endogenous Hairy protein indicated that it is unable to repress a distal enhancer if it binds in a regulatory region lacking activators, possibly because Hairy cannot access a domain that has not been subject to remodeling induced by activators – the so-called "hot chromatin" model [62]. Simulating this situation, we performed chromatin immunoprecipitations in a strain lacking the Gal4 activator protein, and found that the Hairy chimera is able to access the promoter (Figure 3-2A, fourth panel). This result suggests that locally-acting activators were not required to recruit this form of Hairy. However, the promoter context may be more permissive than distal enhancer regions, or the endogenous Hairy protein may be subject to more stringent requirements for DNA-binding. The binding of the repressor did not affect total histone H3 levels, K27 methylation, and had only modest effects on histone acetylation levels (most frequently a slight decrease in relative levels of histone H3 acetylation).

In light of the long-range contacts seen for the Groucho corepressor, we measured histone levels and histone acetylation levels on the coding region of the reporter gene (Figure 3-3). The results showed that changes in acetylation and histone levels associated with repression could be observed up to a distance of 1 kb downstream from the promoter. However, little change was observed at 2 kb and 4 kb downstream. Taken

Figure. 3-3. Change in acetylation levels and histone occupancy is limited to 1 kb from promoter.

Chromatin changes occurring on the transcribed region of the gene during activation, repression, and reactivation assayed by chromatin immunoprecipitation. **(A)** Relative histone H3 K9/14 acetylation levels (normalized to H3) at +1, +2, and +4 kbp. A strong increase in relative H3 acetylation at +1 kbp is observed with Gal4 activator present, and this acetylation decreases after induction of the repressor. No large changes at +2 and +4 kbp were observed. **(B)** Relative histone H4 K5/8/12/16 acetylation levels (normalized to H3) at +1, +2, and +4 kbp. Promoter-proximal acetylation status was observed to increase after activation, and decrease during repression. A smaller decrease in acetylation at +2 and +4 kbp was usually observed. During reactivation, a hyperacetylation of histone H4 was observed in the coding region. **(C)** Total relative histone H3 occupancy on reporter. Overall levels of H3 at the promoter decreased in the activated state relative to the unactivated gene, and increase again during repression. No change was observed at +2 and +4 kb. During reactivation H3 levels decreased throughout the coding region. Shown are the average value and standard deviation of at least 4 biological replicates for the activated and repressed states. Average value of 2 biological replicates is shown for the reactivated state. Signals have been normalized to the unactivated state. Images in this dissertation are presented in color.

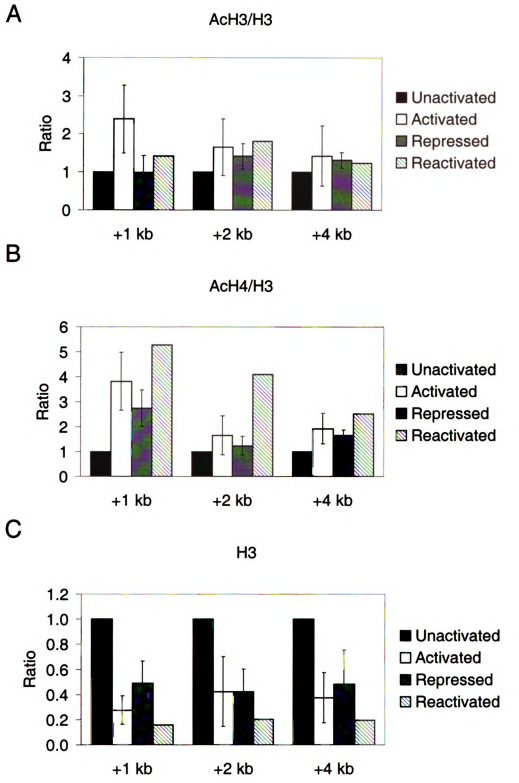

Figure. 3-3. Change in acetylation levels and histone occupancy is limited to 1 kb from promoter.

together, these results suggest that activation and repression of the reporter gene is accompanied by changes in histone occupancy and acetylation, but that the changes induced by activation are not completely reversed by repression.

We also analyzed the changes in chromatin modifications during reactivation of the reporter. Intriguingly, a strong acetylation signal of histone H4 was observed throughout the coding region during reactivation. In addition, total levels of histone H3 dropped throughout the coding region during reactivation. These results suggest that the reactivated state is different then the activated state.

Discussion

Despite the intensive study of Hairy and other HES proteins in development, little is known of the mechanism of this model long-range repressor. Thus, we lack a full understanding of how distinct corepressors may be implicated in different developmental circuitry, or the logic of regulatory regions controlled by this protein. Our finding that Groucho associates with a Hairy-repressed locus over the distance of several kilobases provides a possible mechanism that explains how Hairy can influence the activity of activator sites over a great distance. The molecular nature of the repression mechanism may involve "spreading", as suggested by Courey, involving progressive deacetylation and binding to hypoacetylated histones [17, 244].

In support of this model, Groucho binds hypoacetylated histone H3 and H4 tails, and mutations in its N-terminal oligomerization domain block its repression activity *in vivo* [84, 244]. Recruitment of histone deacetylases may thus enhance Groucho binding to adjacent histones in a positive feedback loop. A similar mechanism has been suggested for Tup1, the Groucho homolog in yeast [85]. As with Groucho, histone deacetylases have been shown to be crucial for Tup1 repression [245]. Moreover, Tup1 also has affinity for hypoacetylated amino-terminal histone tails, and mutations or deletions of the tails cause derepression of Tup1 targets [246, 247]. As with the Hairy repressor here, Tup1 does not change methylation status of target genes, and deletion of histone methyltransferases do not affect Tup1 mediated repression [248]. This suggests that methylation marks may not need to be reversed to achieve repression, but they may facilitate ready reactivation seen upon depletion of Hairy. Regarding the extent of association of Tup1 with target genes in yeast, chromatin immunoprecipitation studies

have yielded conflicting pictures. Tup1 has been reported to interact with the **a**-cell specific *STE6* gene only at the promoter, or spread from 1-3.5 kbp, encompassing the entire gene [249-251]. This discrepancy may be due to differences in crosslinking or immunoprecipitation conditions, reflecting the difficulty in analyzing indirectly bound factors. Indeed, in our study, promoter interactions by Groucho were observed only with the use of a double crosslinking protocol.

The extensive contacts of Groucho over the repressed locus are strongly reminiscent of the extended nucleoprotein structures deposited on regions repressed by stable, heritably acting systems such as Polycomb group (PcG) proteins in animals and Sir proteins in silent mating type loci and subtelomeric regions of yeast. There, chromatin regions are modified and inhibited for formation of productive transcription complexes [80, 252, 253]. Indeed, the association of activators and components of the transcriptional machinery with repressed loci in these systems mirrors the continued binding of activators and coactivators in the system we study here, suggesting that the limiting factor for transcription occurs at a later stage [254-256]. What sort of inhibitory interaction might be involved in this case? A number of recent reports have raised the possibility that repressed, or non-activated promoters, feature RNA polymerase II that is blocked for elongation, similar to the paused polymerase found at the *hsp70* locus under noninducing conditions [257]. Gergen and colleagues find that RNA polymerase II is not displaced from the *slp1* gene upon repression with Runt, a Groucho-binding protein [258]. It is possible that Groucho itself, through contacts with histone proteins, and/or recruitment of deacetylases such as Rpd3, establishes a chromatin environment that is inhibitory for transcriptional elongation. We were unable to obtain reliable signals for RNA polymerase

II at this promoter, precluding a definitive statement about polymerase occupancy in activated and repressed states, however.

A difference between the repression complex assembled by the Hairy repression domain and by these other proteins is the transience of the effect; while PcG regulation is linked to epigenetic modifications that allow repression to persist for an extended time when PcG proteins are depleted [259], the regulation we see here is readily reversed upon loss of the LexA-Hairy repressor. Similar effects are observed with elements regulated by the endogenous Hairy protein; enhancers bearing Dorsal and Twist activator sites that are repressed by Hairy in the blastoderm embryo are reactivated minutes later in the germband extended stage (M. Kulkarni, unpublished). Thus, Hairy appears to be designed for highly effective, but readily reversible, repression, which may be useful in particular developmental settings.

In contrast to a linear spreading model, an alternative picture of Groucho interaction that is consistent with our observations is that the corepressor may be tethered to the promoter region, forming larger multimeric complexes around which proximal and distal portions of the gene are wrapped ("turban" model). This model may explain why we often detect downstream interactions, albeit weaker ones, of the LexA-Hairy repressor, particularly when employing the more extensive double crosslinking protocol. In either case, Groucho itself may be important for interfering with activity of transcription factors, or transcription of distant loci. Both of these models suggest that the extensive spread or extensive contacts of Groucho is mechanistically linked to transcriptional repression. However, it is possible that Groucho's extensive contacts with downstream regions are not the main effector of Hairy-mediated repression. Promoter-

proximal activities of Groucho, or of other Hairy corepressor proteins, may play the decisive role in dictating long-range effects. However, extensive experimental evidence indicates that Groucho plays a key role in repression mediated by Hairy, thus it seems parsimonious to assume that Groucho activity on the repressed gene is important for repression.

Our study demonstrates that repression by Hairy is associated with histone deacetylation, which is certainly consistent with the nature of cofactors associating with this protein. Interestingly, this modification appears to be restricted to regions close to the repressor binding sites, which in our configuration places them close to the transcriptional initiation site. How might this be related to the long-range effects mediated by Hairy? One possibility is that Hairy, regardless of where it is bound, induces characteristic changes on chromatin close to the transcriptional start site, which would induce a dominant (and hence long-range) effect on target genes. Alternatively, the local chromatin deacetylation may reflect the reversal of promoter-localized histone acetylases (e.g. SAGA), and acetylation levels on other portions of the gene are already too low to show robust deacetylation. A third possibility is that other Hairy-induced chromatin modifications that are not assayed here are more extensive than the deacetylation.

Our study strongly supports a model for Hairy repression that involves contacts between the Groucho corepressor and extended regions of the silenced gene, providing a basis for the long-range repression observed for this protein that is independent of activator displacement. An interesting question for future studies is how Groucho spreading is limited, and whether specific chromatin signals modulate this activity. In addition, such extended repression complexes might be specific to subsets of Hairy

targets. Genomic surveys indicate that Hairy is likely to associate with distinct cofactors at different loci; future work will focus on identifying the roles of individual cofactors of this repressor at genes that represent the diversity of Hairy targets in *Drosophila*.

Chapter IV

Conclusions and Future Directions

Development of a model system for the study of transcriptional regulation

Over the last 30 years, numerous studies have sought to understand the molecular mechanism of repression by HES proteins [1, 2, 260, 261]. Functional characterizations of multiple HES proteins have revealed a highly conserved role as regulators of cell fate decisions (reviewed in Chapter1). In development, HES proteins typically function as effectors of the Notch/Delta signaling pathway to negatively regulate genes involved in establishing cellular identity [1, 2]. However, some HES proteins, like *Drosophila* Hairy, are not under the control of Notch signaling and function instead to prepattern the initial set of regulators specifying early pluripotent cellular precursors [2, 262, 263]. Members of the HES family have a bHLH DNA-binding domain, a conserved Orange domain, and a C-terminal WRPW motif that interacts with the corepressor Groucho [1]. The high degree of homology between HES proteins suggests an evolutionarily conserved repression mechanism. A detailed molecular analysis of HES-mediated repression would lead to a deeper understanding of multiple developmental pathways and would provide opportunities for developing novel treatments for diseases found to be associated with disruption of normal HES function.

In this project I developed a highly defined embryo system to study Hairy repression *in vivo*. As discussed in Chapter 2, the use of exogenous activator and repressor transgenes allowed a great degree of control over the system. The spatial and temporal control of the activator and repressor inputs permitted the embryos to be treated

as a uniform collection of cells, analogous to tissue culture studies. Further analytical power was afforded through the creation of alternate *Drosophila* lines carrying either the reporter alone, reporter and activator, reporter and repressor, or all three transgenes. This combinatorial analysis of promoter states proved extremely useful in validating the conclusions presented here, and gave insights into both the transcriptional activation as well as repression processes.

It should be noted that other groups have also succeeded in using whole embryo systems to study the molecular mechanisms of transcriptional regulation [239, 258]. For example, in a recent study, *Drosophila* $Toll^{10b}$ mutant embryos were used to determine the genome wide occupancy of the Twist, Dorsal, and Snail transcription factors [239]. These embryos express a constitutively active form of the Toll receptor leading to translocation of Dorsal into the nucleus and the uniform expression of Twist and Snail [264]. Similarly, overexpression of Ftz and Runt during the cellular blastoderm stage of *Drosophila* development result in the uniform suppression of *sloppy-paired-1* (*slp1*), while overexpression of Ftz and Opa results in uniform activation [258]. However, adapting these model systems to study different activators or repressors would require extensive reengineering of the gene network that makes up the system. In contrast, the embryo system developed here can readily be adapted to investigate the transcription regulatory mechanisms of any protein containing modular repression or activation domains.

While the repression observed in this study takes place in a short-range context, there are two reasons why the conclusions of my study should also be valid in a long-range context. First, the LexA-Hairy protein is able to suppress the activity of a

rhomboid-twist driven reporter even when bound 2 kbp upstream, indicating that it can also function in a long-range context (Li Li, unpublished results). Furthermore, the terms short-range and long-range refer to a functional characterization of the repressor and do not imply any particular molecular mechanism [16, 22]. Thus, the conclusions derived using the model system developed here are relevant to the general mechanism of Hairy repression.

Hairy repression mechanism does not rely on activator or coactivator displacement

Using the highly defined embryo system discussed previously, I was able to show that the repression mechanism of Hairy works even when the activator and coactivator remain bound to the gene (Figure 2-6, 2-7). This result raises the possibility that Hairy repression could involve direct targeting of the basal machinery. Indeed, repression of *slp1* by Ftz and Runt, the latter of which also binds Groucho, leads to the formation of a paused Pol II complex downstream of the promoter [258, 265].

Preliminary experiments using the 8WG16 antibody showed a decrease in Pol II signals at the promoter during LexA-Hairy repression when compared to the activated state (Appendix B). Unexpectedly, the downstream Pol II signals were actually higher in the repressed state than the activated state. A possible explanation for this result is that LexA-Hairy repression could induce a transcriptional block near the 3' end of the gene. Studies in yeast have shown that both Gal4 and SAGA can directly interact with components of the basal machinery and promote PIC assembly even in the absence of Gcn5 HAT activity [181, 182, 186]. The continued presence of Gal4 and SAGA during repression could lead to the observed accumulation of Pol II in the coding region.

Unfortunately, the lack of downstream Pol II signals in the activated state and the poor reproducibility of the Pol II immunoprecipitations prevented any definite conclusions to. Therefore, future studies should focus on determining if Hairy repression involves blocking PIC formation or inhibiting Pol II elongation.

Since Gal4 has been shown to "lock in" and form a highly stable Gal4-promoter complex, it is possible that other activators with weaker DNA affinities could be displaced by Hairy repression [235]. In order to test this possibility, binding sites for endogenous activators could be used instead of Gal4 to drive expression of the reporter construct. Antibodies against the activator of interest (if available) could be used to track activator binding. Alternatively, the activators could be epitope-tagged, and expressed using a ubiquitous driver.

Another possibility is to use the embryo system I have developed to study other transcriptional repressors such as Knirps or Giant. In fact, the original goal of this project was to understand the molecular mechanisms differentiating short- from long-range repression by tethering a Knirps or Hairy repression domain to LexA and using the *lacZ* reporter shown in Figure 2-1. While both LexA-Knirps and LexA-Hairy could repress the reporter described in Chapter 2 (Figure 2-2), only LexA-Hairy could suppress gene activation in the embryo system (Figure 2-1, Appendix A). This result was unexpected, since it had previously been shown that the short-range repressor Giant can repress a synthetic enhancer with a similar arrangement of activator and repressor binding sites (2 Giant, 5 UAS, 2 Giant) [229]. Since Knirps has been shown to be able to suppress the activity of three high affinity UAS sites, the inability of LexA-Knirps to repress five tandem UAS sites is not due to repressor-activator specificity [230]. Perhaps Giant is a

stronger repressor then Knirps, and that is why it is able to repress 5 UAS sites. Indeed, a comparison of the relative potency of the short-range repressors Giant, Knirps, and Kruppel, in the context of three UAS sites, showed that in general Knirps has a lower activity [230].

As discussed in the introduction, short-range repressors allow enhancer modularity by blocking those activators bound within 150 bp from its target sequence [18]. One possible mechanism by which short-range repressors could block nearby activators is by displacing locally bound activators from the DNA. By modifying the embryo system to include different short-range repressor domains, this hypothesis could be directly tested. A recent genome-wide study looking at the binding of the short-range repressor Snail, and the Twist and Dorsal activators show that all three proteins can bind simultaneously to inactive enhancers, suggesting that short-range repressors also do not displace activators [239].

Hairy repression is associated with Groucho binding to the entire gene locus

A key finding in this project is that the Hairy repression mechanism is associated with Groucho binding to a wide region encompassing the gene (Figure 3-1). This result provides strong evidence for a model, first proposed by Al Courey, that Hairy long-range repression is mediated by a Groucho spreading mechanism, similar to the spreading of the Sir2 complex in yeast (Fig 4-1) [17, 79]. A similar mechanism has also been proposed for the yeast homolog Tup1, but evidence of Tup1 spreading has been controversial [249-251]. Like Tup1, Groucho has an affinity for hypoacetylated histones,

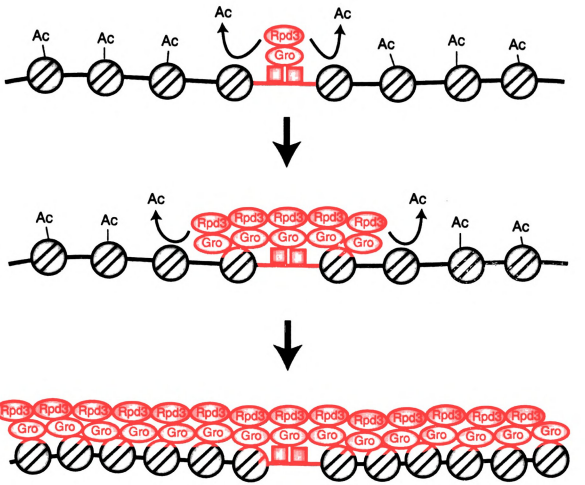

Figure 4-1. Groucho spreading model

The model for Groucho spreading involves: 1) recruitment of Groucho by a long-range repressor such as Hairy, 2) deacetylation of the surrounding histones by the associated Rpd3 histone deacetylase, 3) Groucho binding to adjancent nucleosomes through interactions with the hypoacetylated H3 and H4 histone tails. Images in this dissertation are presented in color.

suggesting a model wherein Groucho-mediated Rpd3 recruitment deacetylates surrounding histones; local histone deacetylation in turn promotes further Groucho spreading [17, 266]. The association of Groucho/Rpd3 to large tracts of the gene could repress transcription by catalyzing the formation of a heterochromatin-like structure. Interestingly, Sir2-dependent heterochromatic silencing of the yeast *Hsp82* gene does not prevent the HSF activator, or TBP, from binding to the promoter, suggesting possible structural similarities between Sir2- and Groucho-remodeled chromatin [254]. In support of the importance of Groucho spreading for long-range repression, mutations in the Groucho oligomerization domain abolish its repressive activity [244].

Since the extensive Groucho binding observed in this study is in the context of an artificial model system, future studies should focus on ascertaining the relevance of Groucho spreading, by looking at Groucho binding to endogenous enhancers. A possible approach could be to chromatin immunoprecipitate Groucho from embryos or tissue culture cells, followed by hybridization to a tiled microarray (ChIP-chip), or by direct sequencing of the immunoprecipitated DNA (ChIP-PET) [267].

As discussed in Chapter 3, this study raises the question of what regulates Groucho spreading so that it does not interfere with the transcriptional activation of nearby genes. Future studies should focus on elucidating the mechanism by which Groucho spreading is limited and whether histone modifications have any role in regulating this activity. Additionally, analysis of LexA-Hairy repression in a long-range context could determine if Groucho spreading is limited to the basal promoter and ORF, or if it can also spread through upstream *cis* regulatory regions. A related question regarding Hairy repression is whether boundary elements can block Groucho spreading.

For example, the Su(Hw) insulator protein can partially suppress heterochromatin spreading in *Drosophila* [268]. Similarly, the vertebrate insulator element cHS4 functions to prevent heterochromatin silencing in the chicken β-globin locus [269]. This type of mechanism might account for why the Su(Hw) insulator can block Hairy repression of a downstream enhancer while allowing closely linked activators to stimulate transcription [22].

In addition to Groucho, Hairy has also been found to associate with the Sir2 and CtBP corepressors [69, 71]. Future studies should address the role of these cofactors in mediating Hairy long-range repression. Of particular interest would be to understand the role of Sir2, because of its critical involvement in heterochromatin silencing. A genome-wide survey of Hairy, Groucho, CtBP, and Sir2 binding, found that Hairy associates with different combinations of cofactors at target loci, suggesting that Hairy cofactor recruitment is context dependent [75]. Interestingly, only a small percentage of putative Hairy targets were associated with Groucho, with the majority associated with CtBP and Sir2 [75].

A possible explanation for the apparent context dependency of cofactor recruitment is that the chromatin structure regulates cofactor binding. Indeed, Groucho was found to bind preferentially to hypoacetylated histone H3 [85]. Similarly, the Sir2 complex preferentially binds deacetylated histones through the Sir3 and Sir4 subunits [270]. However, initial binding of Sir2 does not require HDAC activity, suggesting that it is not dependent on histone acetylation [271]. The role of chromatin in regulating cofactor recruitment could be investigated by analyzing cofactor binding to the *lacZ* reporter in the context of the reporter alone, reporter + activator, reporter + repressor, and

reporter + repressor + activator, since each of these states is associated with a specific chromatin structure. Binding of both LexA-Hairy and Gal4 to the promoter was associated with a marked decrease in the acetylation level (Figure 3-2B). In contrast, binding of LexA-Hairy alone has only a slight effect on H4 acetylation levels, suggesting that in this context LexA-Hairy does not recruit a cofactor that mediates this activity (Figure 3-2A, compare panels 3 and 4).

Hairy repression is associated with histone deacetylation and chromatin remodeling

It has been shown that Hairy associates with histone deacetylases [71, 84], consistent with this finding, this study provides direct evidence of HDAC recruitment and subsequent histone deacetylation by Hairy. As can be seen in Fig 3-1, the Rpd3 HDAC was shown to track with Groucho up to 2 kbp downstream of the transcriptional start site. Curiously, LexA-Hairy binding to the promoter resulted only in local histone H3 deacetylation up to a distance of 1 kbp (Figure 3-3A, B). However, it should be noted that even in the unrepressed state, the histone H3 acetylation signals from the 3' end of the *lacZ* reporter decreased dramatically relative to the promoter, making the task of detecting changes in histone acetylation more difficult (Figure 3-3A, B). Furthermore, there is a 5' to 3' decrease of Groucho and Rpd3 signals, implying a smaller reduction in acetylated histone levels.

In this project I have been able to show that chromatin remodeling is associated with Gal4 activation as well as Hairy repression. Comparisons of histone H3 signals between the unactivated and activated state revealed a marked decrease in H3 promoter occupancy during Gal4-mediated activation (Figure 3-2B). This result is consistent with

previous studies that Gal4 activation is associated with the loss of promoter-proximal histones in yeast [212]. Importantly, during LexA-Hairy repression there was a reproducible increase in promoter bound H3 signals. In yeast, repression by the Groucho homolog Tup1 has also been shown to involve nucleosome positioning, suggesting a conserved repression mechanism [272]. In order to have a better understanding of Hairy-dependent chromatin remodeling, micrococcal nuclease digestion can be used to determine if repression involves nucleosome positioning such as that seen for a subset of Tup1-Ssn6 repressed genes [272].

Gal4 activation is associated with demethylation of K27

Since K27 methylation is a mark of heterochromatin, I analyzed whether Hairy repression was associated with K27 methylation. To this end, chromatin immunoprecipitation assays were carried out using antibodies against dimethylated H3 K27. The result showed that this modification was not associated with Hairy-mediated repression. Instead, K27 methylation was correlated with Gal4 activation (Figure 3-2B). Future studies should continue to investigate the possible involvement of additional histone marks such as H2B ubiquitylation. An exhaustive analysis of histone modification marks would most likely yield additional insights into Hairy repression activity.

Reactivation of gene activity after LexA-Hairy repression

Using the embryo system, I was able to show that induction of the LexA-Hairy fusion protein led to transient repression of the *lacZ* reporter construct (Figure 2-4). In

contrast, other long-range repression mechanisms such as those mediated by the Polycomb, HP1, and Sir proteins, lead to stable gene silencing [16]. Reactivation of the *lacZ* reporter was accompanied by a corresponding increase in histone H3 and H4 acetylation levels (Figure 3-2, 3-3). Unexpectedly, during reactivation H4 acetylation levels in the coding region were higher than during the activated state (Figure 3-3B). One possible model that could help explain the high H4 acetylation levels observed is that Groucho/Rpd3 spreading primes the gene for reactivation. Studies in yeast have shown that the Rpd3S HDAC complex is targeted to the open reading frame of active genes by binding to methylated H3 K36 histones [273]. The Rpd3S subunit Eaf3 targets the complex through its chromodomain which specifically recognizes methylated H3 K36 [274]. Since the Eaf3 protein is also shared with the NuA4/Tip60 complex that catalyzes H4 acetylation, it suggests a reactivation model wherein the Rpd3S HDAC complex is exchanged for the NuA4/Tip60 HAT complex, leading to generalized H4 hyperacetylation in the ORF [275]. Therefore, future studies could address whether Groucho/Rpd3 spreading promotes NuA4/Tip60 recruitment during reactivation.

An alternative reactivation model involves Groucho-mediated deposition of the conserved H2A.Z histone variant. In yeast, the SWR1 chromatin remodeling complex has been shown to catalyze the exchange of H2A with the H2A.Z histone variant [276, 277]. H2A.Z deposition functions to prevent heterochromatin spreading and to promote efficient reactivation at repressed promoters [278-280]. Importantly, the Groucho homolog Tup1 promotes the deposition of H2A.Z on the *GAL1* promoter nucleosome, suggesting that it primes the promoter for reactivation [281]. Since NuA4 and SWR1 share multiple subunits and are both conserved in *Drosophila* [282], I propose a

reactivation model wherein Groucho/Rpd3 spreading induces recruitment of SWR1 and NuA4 to the coding region (Figure 4-2). Recruitment of SWR1 and NuA4 could in turn catalyze the deposition of H2A.Z and hyperacetylate H4, respectively leading to a looser chromatin structure and facilitating reactivation. In order to test this model, H2A.Z recruitment during repression and reactivation can be determined.

Contrasting transcriptional states: Unactivated versus Repressed

An important observation of my study is that repression does not reset the chromatin structure to the unactivated state. As can be seen in Figure 3-2B, in the unactivated state there is a high level of methylated H3 K27 histones, suggesting that the reporter gene is in a heterochromatic environment. Gal4-mediated activation leads to a reduction of methylated K27, relative to H3, consistent with an activation mechanism that remodels chromatin from a heterochromatic silenced state to a euchromatic active state. Heat shock induction of LexA-Hairy leads to the suppression of *lacZ* expression, yet it does not reverse the loss of K27 methylation seen during activation. Thus, the unactivated transcriptional state is not equivalent to the repressed state.

Figure 4-2. Proposed mechanism for Groucho-dependent reactivation

During repression Groucho and Rpd3 are found associated with the entire coding region. H3 K36 methylation stabilizes the Rpd3 binding through direct interactions with the chromodomain-containing Eaf3 subunit. Since the Eaf3 subunit is shared between Rpd3 and NuA4, reactivation could occur by a subunit exchange that allows NuA4 to be recruited to the coding region. Subsequently, NuA4 can catalyze the hyperacetylation of H4, creating a looser chromatin structure. NuA4 and the ATP-dependent chromatin remodeling complex SWR1 share four subunits. SWR1 can catalyze the exchange of H2A with H2A.Z to prime the gene for reactivation. Images in this dissertation are presented in color.

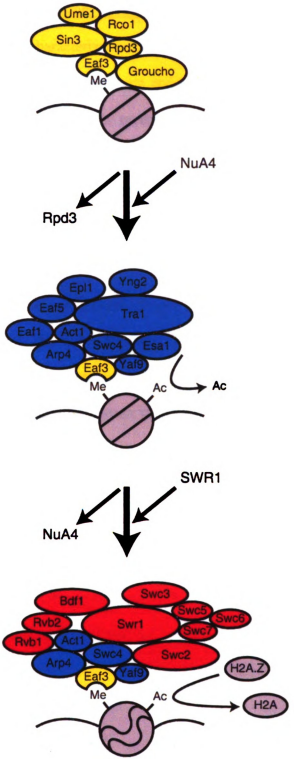

Figure 4-2. Proposed mechanism for Groucho-dependent reactivation

Short versus long-range repression: Two sides of the same coin

Previous functional characterizations of repressors in *Drosophila* pointed to a role of CtBP in mediating short-range repression and a role of Groucho in mediating long-range repression [72-74]. However, a recent study suggests that Groucho-mediated repression can have a short-range activity [62, 283]. Additionally, biochemical purification of the short-range repressor Knirps indicated that Groucho is a part of the repressor complex (Sandhya Payankaulam, unpublished results). Similarly, the CtBP cofactor which potentiates short-range repressors such as Knirps, has been found to associate with the Hairy long-range repressor and to be important for the repression of putative Hairy targets [69, 75]. Taken together, the evidence suggests that Groucho and CtBP form part of a common repression pathway. Indeed, some repressors like the *Drosophila* Hairless and Brinker have been shown to require both CtBP and Groucho to for their activity [284, 285].

The fact that Groucho has been shown to be important for Hairy long-range repression raises the question of what prevents Groucho-mediated long-range repression in a short-range context [72]. Perhaps the range of Groucho repression is regulated by limiting the degree to which Groucho can spread in chromatin. One possibility is that the extent of Groucho spreading is determined by the total number of repressor binding sites present. In support of this model is the fact that the range of Hairy repression increases with additional Hairy sites [22]. Alternatively, the distance to which Groucho can spread could depend on the chromatin context. An intriguing idea is that Groucho spreading could be limited by the same mechanisms that limit the spreading of silent heterochromatin.

In yeast, there are several chromatin-based mechanisms that prevent the Sir2 complex from spreading into euchromatic regions [286]. The first is through site-specific acetylation of H4 K16 by the Esa1 and Sas2 histone acetyltransferases [287, 288]. This modification has been shown to be specifically enriched at heterochromatin-euchromatin boundaries [289]. A second mechanism involves the exchange of H2A for the H2A.Z histone variant catalyzed by the SWR1 complex [282]. Deposition and subsequent NuA4-mediated acetylation of H2A.Z has been shown to be required for the formation of heterochromatin borders [279, 290]. A third mechanism involves the creation of nucleosome free regions [291]. In yeast, heterochromatic spreading from the silent mating type locus HMR is blocked by the neighboring $tRNA^{Thr}$ gene, which is partially depleted of histones [292]. Recently, it has been reported that Set2 methylation of H3 K36 can also prevent heterochromatin spreading [293]. A combination of these mechanisms could account for the observed differences in the range of Groucho repression.

Contextual dependency of transcriptional repression: Implications for modeling *cis* regulatory elements

One of the most pressing problems in the post-genome era is trying to develop a conceptual framework that can be used to find and decode *cis* regulatory elements. The *Drosophila* embryo has become an ideal model system because many of the transcription factors and *cis* regulatory elements involved in early embryogenesis have been characterized [294]. Currently, efforts are being made by several labs to develop transcription regulatory models that can predict gene expression patterns directly from

the DNA sequence [294-296]. An underlying assumption of these efforts is that the *cis* regulatory elements under study are flexible with respect to their binding site arrangement, with more then one "solution" leading to a particular gene expression pattern [297]. Comparative phylogenetic analysis of the *eve* stripe2 enhancer supports this idea, as the stripe 2 enhancers from four different *Drosophila* species show identical stripe placement despite varying widely in binding site arrangement [298]. However, if the range of Groucho repression is context dependent, it would place restrictions on the evolution of *cis* regulatory elements that recruit Groucho-associated repressors.

The fact that Groucho-dependent repression can act in a short- or long-range manner, suggests that new design principles are needed to model enhancer activity. Previous work from the Arnosti lab sought to discover "grammatical" rules governing the function and design of modular enhancers [229, 299]. As a result, four basic parameters were determined to be critical for modeling short-range transcriptional repression: 1) total number of repressor binding sites, 2) repressor-activator stoichiometry, 3) binding site affinity, and 4) binding site arrangement [229]. In order to extend the applicability of this approach to include Groucho-associated repressors, additional grammatical rules must be implemented. For example, if we assume that Groucho spreading, like Sir2, is blocked by H4 K16 acetylation, then activators that recruit the NuA4/Tip60 HAT, which mediates this modification, could result in short-range repression by Groucho-associated factors. In addition, sequences that tend to exclude nucleosome binding could also have an impact on the type of repression activity. Therefore, future studies should focus on discovering novel grammatical rules that take into account those parameters that influence the range of repression.

Concluding remarks

In summary, I have presented strong evidence that Hairy long-range repression is mediated by a mechanism involving Groucho spreading and chromatin remodeling. In the literature, there are several examples of similar long-range repression mechanisms. However, a key feature that differentiates the repression mechanism studied here from previously characterized long-range mechanisms is the easy reversibility of the repression. The transient nature of repression could be a reflection of an evolutionarily conserved mechanism whereby the corepressor Groucho primes the gene for reactivation. Efficient reactivation of HES target genes could be of critical importance for the proper regulation of developmental pathways. For example, the role of HES proteins as part of the "segmentation clock" requires repetitive repression-reactivation cycles in order to generate waves of transcriptional activity [140, 145]. Inefficient reactivation of HES target genes could lead to uncoordinated patterns of gene expression and disruption of the segmentation process.

In addition, I have succeeded in developing and characterizing a highly defined embryo system that can serve as a powerful platform for future studies of Hairy long-range repression. Such studies could lead to a better understanding of the molecular mechanisms by which cell fate decisions are regulated. Furthermore, the different *Drosophila* lines I have created, capable of producing alternative promoter states, can be used to provide novel insights into how the chromatin context affects transcriptional repressor activity. A better understanding of the role of chromatin in regulating the range of transcriptional repression is essential in order to develop predictive quantitative models of enhancer function.

APPENDIX A

Characterization analysis of the LexA-Knirps fusion protein

One of the initial objectives of the project was to understand the molecular mechanism of short-range repression. To this end, a fusion construct of LexA and Knirps was tested for its ability to repress in the embryo system and in a rhomboid-twist driven reporter. The repression domain used for Knirps encompassed the amino acids 75-429. This domain had previously been shown to be sufficient to mediate short-range repression of a heterologous stripe2 enhancer when tethered by the Gal4 DNA-binding domain [300]. The repression domain contains the CtBP PXDLS binding motif near the C-terminus but lacks the endogenous DNA-binding domain. Previously, it had been shown that a *lacZ* reporter containing tandem Knirps sites placed 5' and 3' of three Gal4 UAS sites was repressed in the region of Knirps expression [229].

In order to test the repression activity of LexA-Knirps, a *Krüppel* promoter was used to drive expression in a central band of the embryo. A transgenic line carrying the LexA-Knirps construct was crossed to both the embryo system (carrying only the Gal4 activator and reporter), and a rhomboid-twist driven reporter with LexA sites near the promoter. Using in situ hybridizations against lacZ, repression was observed only in the rhomboid-twist reporter but not in the embryo system (Figure A-1).

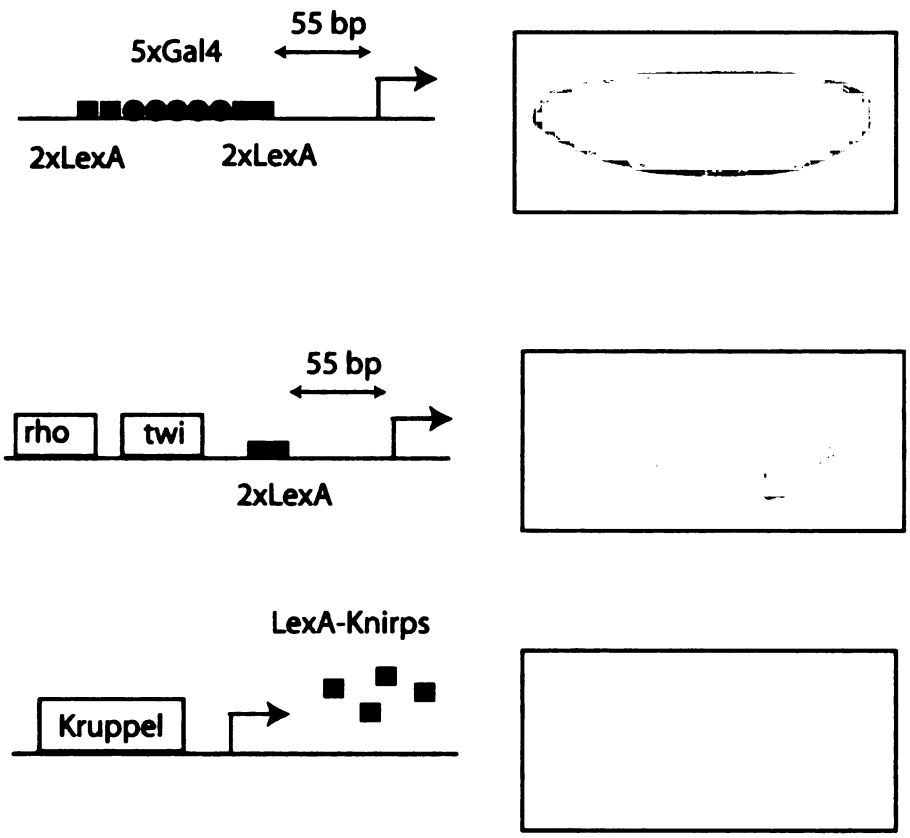

Figure A-1. LexA-Knirps does not repress in the model system

Top panel: A line containing a Kruppel driver expressing the LexA-Knirps (75-429) was crossed to the defined embryo system; at left is a schematic diagram of the reporter construct. Middle panel: LexA-Knirps mediates efficient repression of a rhomboid-twist driven reporter when bound within 55 bp of the promoter. Bottom panel: Antibody staining against LexA shows that LexA-Knirps is being expressed in these embryos. Images in this dissertation are presented in color.

Materials and Methods

Plasmids. The reporter constructs were constructed as described in Chapter 2. The LexA-Knirps fusion construct was made by joining a *Bam*HI/*Kpn*I fragment containing a Kozak sequence, initiator ATG, and coding sequence for the entire LexA protein (amino acid residues 1-202) in frame to a *Kpn*I/*Xba*I fragment containing the Knirps repression domain (residues 75-429). The fusion construct was placed in a modified pKreg vector containing the Kruppel driver. The resulting plasmids were introduced into the Drosophila germline by P-element transformations as described [231].

***In situ* hybridization and antibody staining of *Drosophila* embryos.** Embryos were fixed for *in situ* hybridization and stained using a digoxigenin-UTP labeled antisense RNA probe to *lacZ* as described [231]. Antibody staining for LexA-Hairy expression was done using a 1:2:1 mixture of three mouse monoclonal antibodies raised against LexA, YN-lexA-2-12 (2 μg/ml, 1:2100), YN-lexA-6-10 (4 μg/ml, 1:2023), YN-lexA-16-7 (4 μg/ml, 1:3950) obtained as a gift from Steve Triezenberg [233]. Quantitation of *lacZ* staining was done by scoring the stained embryos into unstained, lightly stained, and darkly stained from 10-13 random fields. Total embryos scored per slide varied between 135 and 457.

APPENDIX B

Pol II accumulates in the ORF during repression

As discussed in Chapter 4, one of the possible mechanisms of Hairy repression could be blocking Pol II binding or elongation. Using the embryo system, I collected embryos carrying either the reporter and activator; or the reporter, activator, and repressor transgenes. After a 20 min heatshock and 30 min recovery a chromatin immunoprecipitation assay against the Pol II CTD revealed a loss of Pol II from the promoter during repression. At the same time there was an increase of Pol II in the open reading frame, suggesting that Hairy repression does not suppress Pol II recruitment. Instead, repression could involve a block to elongation near the 3' end of the gene.

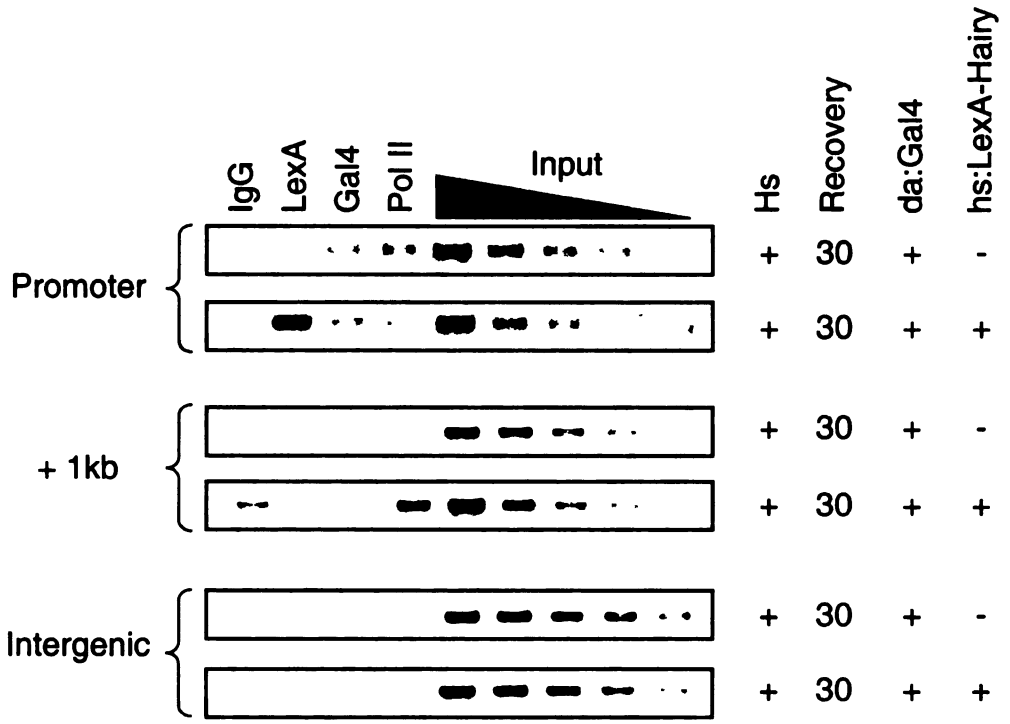

Figure B-1. Chromatin immunoprecipitation of Pol II.

Embryos expressing only the activator or the repressor were collected and heatshocked for 20 min at 37°C and recovered for 30 min at room temperature. Activated embryos showed a strong Pol II signal at the promoter but no Pol II at + 1 kb. During repression Pol II signals decreased slightly at the promoter but increased at +1 kb. PCR using primers against an intergenic region of chromosome 3 did not give any signals indicating that the signals observed are specific. Presence or absence of Gal4, and the heatshock-inducible LexA-Hairy transgene noted by +/-. Input titration was 10%, 2%, 1%, 0.5%, and 0.25%. Images in this dissertation are presented in color.

References

1. Davis, R.L. and D.L. Turner, *Vertebrate hairy and Enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning.* Oncogene, 2001. **20**(58): p. 8342-57.

2. Fisher, A. and M. Caudy, *The function of hairy-related bHLH repressor proteins in cell fate decisions.* Bioessays, 1998. **20**(4): p. 298-306.

3. Carroll, S.B., A. Laughon, and B.S. Thalley, *Expression, function, and regulation of the hairy segmentation protein in the Drosophila embryo.* Genes Dev, 1988. **2**(7): p. 883-90.

4. Struhl, K., *Yeast transcriptional regulatory mechanisms.* Annu Rev Genet, 1995. **29**: p. 651-74.

5. Wolpert, L., et al., *Principles of Development.* Oxford University Press, 1998.

6. Campos-Ortega, J.A. and V. Hartenstein, *The embryonic development of Drosophila melanogaster.* 2nd Edition. Springer, 1997.

7. Veraksa, A., M. Del Campo, and W. McGinnis, *Developmental patterning genes and their conserved functions: from model organisms to humans.* Mol Genet Metab, 2000. **69**(2): p. 85-100.

8. Small, S., *Mechanisms of segmental pattern formation in Drosophila melanogaster Reproductive biology of invertebrates.* In Reproductive biology of invertebrates. K.G. Adiyodi and R.G. Adiyodi, eds. John Wiley and Sons, USA, 1997. **VII**: p. 137-178.

9. Nusslein-Volhard, C. and E. Wieschaus, *Mutations affecting segment number and polarity in Drosophila.* Nature, 1980. **287**(5785): p. 795-801.

10. Sanson, B., *Generating patterns from fields of cells. Examples from Drosophila segmentation.* EMBO Rep, 2001. **2**(12): p. 1083-8.

11. Gray, S., et al., *Transcriptional repression in the Drosophila embryo.* Philos Trans R Soc Lond B Biol Sci, 1995. **349**(1329): p. 257-62.

12. Fujioka, M., et al., *Analysis of an even-skipped rescue transgene reveals both composite and discrete neuronal and early blastoderm enhancers, and multi-stripe positioning by gap gene repressor gradients.* Development, 1999. **126**(11): p. 2527-38.

13. Johnson, A.D., *The price of repression.* Cell, 1995. **81**(5): p. 655-8.

14. Alberts, B., et al., *Molecular Biology of the Cell.* New York, Garland., 1983: p. 440.

15. Herschbach, B.M. and A.D. Johnson, *Transcriptional repression in eukaryotes.* Annu Rev Cell Biol, 1993. **9**: p. 479-509.

16. Gaston, K. and P.S. Jayaraman, *Transcriptional repression in eukaryotes: repressors and repression mechanisms.* Cell Mol Life Sci, 2003. **60**(4): p. 721-41.

17. Courey, A.J. and S. Jia, *Transcriptional repression: the long and the short of it.* Genes Dev, 2001. **15**(21): p. 2786-96.

18. Gray, S., P. Szymanski, and M. Levine, *Short-range repression permits multiple enhancers to function autonomously within a complex promoter.* Genes Dev, 1994. **8**(15): p. 1829-38.

19. Arnosti, D.N., et al., *The gap protein knirps mediates both quenching and direct repression in the Drosophila embryo.* Embo J, 1996. **15**(14): p. 3659-66.

20. Gray, S. and M. Levine, *Short-range transcriptional repressors mediate both quenching and direct repression within complex loci in Drosophila.* Genes Dev, 1996. **10**(6): p. 700-10.

21. Hewitt, G.F., et al., *Transcriptional repression by the Drosophila giant protein: cis element positioning provides an alternative means of interpreting an effector gradient.* Development, 1999. **126**(6): p. 1201-10.

22. Barolo, S. and M. Levine, *hairy mediates dominant repression in the Drosophila embryo.* Embo J, 1997. **16**(10): p. 2883-91.

23. Cai, H.N., D.N. Arnosti, and M. Levine, *Long-range repression in the Drosophila embryo.* Proc Natl Acad Sci U S A, 1996. **93**(18): p. 9309-14.

24. Roseman, R.R., et al., *Long-range repression by multiple polycomb group (PcG) proteins targeted by fusion to a defined DNA-binding domain in Drosophila.* Genetics, 2001. **158**(1): p. 291-307.

25. Jenkins, H.L. and C.A. Spencer, *RNA polymerase II holoenzyme modifications accompany transcription reprogramming in herpes simplex virus type 1-infected cells.* J Virol, 2001. **75**(20): p. 9872-84.

26. Arnosti, D.N., *Multiple Mechanisms of Transcriptional Repression in Eukaryotes.* In M. Gossen, J. Kaufmann, and S. J. Triezenberg (ed.), Handbook of experimental pharmacology: transcription factors, 2004. **166**: p. 33-67. Springer, Heidelberg.

27. Brooks, C.L. and W. Gu, *Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation.* Curr Opin Cell Biol, 2003. **15**(2): p. 164-71.

28. Martinez-Balbas, M.A., et al., *Regulation of E2F1 activity by acetylation.* Embo J, 2000. **19**(4): p. 662-71.

29. Carrozza, M.J., et al., *Gal80 confers specificity on HAT complex interactions with activators.* J Biol Chem, 2002. **277**(27): p. 24648-52.

30. Karin, M. and Y. Ben-Neriah, *Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity.* Annu Rev Immunol, 2000. **18**: p. 621-63.

31. Jaynes, J.B. and P.H. O'Farrell, *Activation and repression of transcription by homoeodomain-containing proteins that bind a common site.* Nature, 1988. **336**(6201): p. 744-9.

32. Kuchin, S. and M. Carlson, *Functional relationships of Srb10-Srb11 kinase, carboxy-terminal domain kinase CTDK-I, and transcriptional corepressor Ssn6-Tup1.* Mol Cell Biol, 1998. **18**(3): p. 1163-71.

33. Zaman, Z., et al., *Interaction of a transcriptional repressor with the RNA polymerase II holoenzyme plays a crucial role in repression.* Proc Natl Acad Sci U S A, 2001. **98**(5): p. 2550-4.

34. Green, S.R. and A.D. Johnson, *Promoter-dependent roles for the Srb10 cyclin-dependent kinase and the Hda1 deacetylase in Tup1-mediated repression in Saccharomyces cerevisiae.* Mol Biol Cell, 2004. **15**(9): p. 4191-202.

35. Li, C. and J.L. Manley, *Even-skipped represses transcription by binding TATA binding protein and blocking the TFIID-TATA box interaction.* Mol Cell Biol, 1998. **18**(7): p. 3771-81.

36. Sauer, F., et al., *Control of transcription by Kruppel through interactions with TFIIB and TFIIE beta.* Nature, 1995. **375**(6527): p. 162-4.

37. Luger, K., et al., *Crystal structure of the nucleosome core particle at 2.8 A resolution.* Nature, 1997. **389**(6648): p. 251-60.

38. Schalch, T., et al., *X-ray structure of a tetranucleosome and its implications for the chromatin fibre.* Nature, 2005. **436**(7047): p. 138-41.

39. Tremethick, D.J., *Higher-order structures of chromatin: the elusive 30 nm fiber.* Cell, 2007. **128**(4): p. 651-4.

40. Razin, S.V., et al., *Chromatin domains and regulation of transcription.* J Mol Biol, 2007. **369**(3): p. 597-607.

41. Comings, D.E., *Mechanisms of chromosome banding. IV. Optical properties of the Giemsa dyes.* Chromosoma, 1975. **50**(1): p. 89-110.

42. Brower-Toland, B., et al., *Specific contributions of histone tails and their acetylation to the mechanical stability of nucleosomes.* J Mol Biol, 2005. **346**(1): p. 135-46.

43. Chandy, M., et al., *SWI/SNF displaces SAGA-acetylated nucleosomes.* Eukaryot Cell, 2006. **5**(10): p. 1738-47.

44. Kouzarides, T., *Chromatin modifications and their function.* Cell, 2007. **128**(4): p. 693-705.

45. Hooper, K.L., S.M. Parkhurst, and D. Ish-Horowicz, *Spatial control of hairy protein expression during embryogenesis.* Development, 1989. **107**(3): p. 489-504.

46. Lardelli, M. and D. Ish-Horowicz, *Drosophila hairy pair-rule gene regulates embryonic patterning outside its apparent stripe domains.* Development, 1993. **118**(1): p. 255-66.

47. Ohsako, S., et al., *Hairy function as a DNA-binding helix-loop-helix repressor of Drosophila sensory organ formation.* Genes Dev, 1994. **8**(22): p. 2743-55.

48. Knust, E., et al., *The enhancer of split locus and neurogenesis in Drosophila melanogaster.* Dev Biol, 1987. **122**(1): p. 262-73.

49. de Celis, J.F., et al., *Functional relationships between Notch, Su(H) and the bHLH genes of the E(spl) complex: the E(spl) genes mediate only a subset of Notch activities during imaginal development.* Development, 1996. **122**(9): p. 2719-28.

50. Sasai, Y., et al., *Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split.* Genes Dev, 1992. **6**(12B): p. 2620-34.

51. Ishibashi, M., et al., *Molecular characterization of HES-2, a mammalian helix-loop-helix factor structurally related to Drosophila hairy and Enhancer of split.* Eur J Biochem, 1993. **215**(3): p. 645-52.

52. Wrischnik, L.A. and C.J. Kenyon, *The role of lin-22, a hairy/enhancer of split homolog, in patterning the peripheral nervous system of C. elegans.* Development, 1997. **124**(15): p. 2875-88.

53. Oates, A.C. and R.K. Ho, *Hairy/E(spl)-related (Her) genes are central components of the segmentation oscillator and display redundancy with the Delta/Notch signaling pathway in the formation of anterior segmental boundaries in the zebrafish.* Development, 2002. **129**(12): p. 2929-46.

54. Dawson, S.R., et al., *Specificity for the hairy/enhancer of split basic helix-loop-helix (bHLH) proteins maps outside the bHLH domain and suggests two*

separable modes of transcriptional repression. Mol Cell Biol, 1995. **15**(12): p. 6923-31.

55. Paroush, Z., et al., *Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins.* Cell, 1994. **79**(5): p. 805-15.

56. Kang, S.A., J.H. Seol, and J. Kim, *The conserved WRPW motif of Hes6 mediates proteasomal degradation.* Biochem Biophys Res Commun, 2005. **332**(1): p. 33-6.

57. Alifragis, P., et al., *A network of interacting transcriptional regulators involved in Drosophila neural fate specification revealed by the yeast two-hybrid system.* Proc Natl Acad Sci U S A, 1997. **94**(24): p. 13099-104.

58. Taelman, V., et al., *Sequences downstream of the bHLH domain of the Xenopus hairy-related transcription factor-1 act as an extended dimerization domain that contributes to the selection of the partners.* Dev Biol, 2004. **276**(1): p. 47-63.

59. Ish-Horowicz, D. and S.M. Pinchin, *Pattern abnormalities induced by ectopic expression of the Drosophila gene hairy are associated with repression of ftz transcription.* Cell, 1987. **51**(3): p. 405-15.

60. Jimenez, G., S.M. Pinchin, and D. Ish-Horowicz, *In vivo interactions of the Drosophila Hairy and Runt transcriptional repressors with target promoters.* Embo J, 1996. **15**(24): p. 7088-98.

61. Van Doren, M., et al., *Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of achaete.* Genes Dev, 1994. **8**(22): p. 2729-42.

62. Nibu, Y., H. Zhang, and M. Levine, *Local action of long-range repressors in the Drosophila embryo.* Embo J, 2001. **20**(9): p. 2246-53.

63. Takebayashi, K., et al., *Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. Negative autoregulation through the multiple N box elements.* J Biol Chem, 1994. **269**(7): p. 5150-6.

64. Hoshijima, K., et al., *Transcriptional regulation of the Sex-lethal gene by helix-loop-helix proteins.* Nucleic Acids Res, 1995. **23**(17): p. 3441-8.

65. Ip, Y.T., et al., *The dorsal morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in Drosophila.* Cell, 1991. **64**(2): p. 439-46.

66. Jennings, B.H., D.M. Tyler, and S.J. Bray, *Target specificities of Drosophila enhancer of split basic helix-loop-helix proteins.* Mol Cell Biol, 1999. **19**(7): p. 4600-10.

67. Jimenez, G., Z. Paroush, and D. Ish-Horowicz, *Groucho acts as a corepressor for a subset of negative regulators, including Hairy and Engrailed.* Genes Dev, 1997. **11**(22): p. 3072-82.

68. Fisher, A.L., S. Ohsako, and M. Caudy, *The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain.* Mol Cell Biol, 1996. **16**(6): p. 2670-7.

69. Poortinga, G., M. Watanabe, and S.M. Parkhurst, *Drosophila CtBP: a Hairy-interacting protein required for embryonic segmentation and hairy-mediated transcriptional repression.* Embo J, 1998. **17**(7): p. 2067-78.

70. Phippen, T.M., et al., *Drosophila C-terminal binding protein functions as a context-dependent transcriptional co-factor and interferes with both mad and groucho transcriptional repression.* J Biol Chem, 2000. **275**(48): p. 37628-37.

71. Rosenberg, M.I. and S.M. Parkhurst, *Drosophila Sir2 is required for heterochromatic silencing and by euchromatic Hairy/E(Spl) bHLH repressors in segmentation and sex determination.* Cell, 2002. **109**(4): p. 447-58.

72. Zhang, H. and M. Levine, *Groucho and dCtBP mediate separate pathways of transcriptional repression in the Drosophila embryo.* Proc Natl Acad Sci U S A, 1999. **96**(2): p. 535-40.

73. Nibu, Y., H. Zhang, and M. Levine, *Interaction of short-range repressors with Drosophila CtBP in the embryo.* Science, 1998. **280**(5360): p. 101-4.

74. Nibu, Y., et al., *dCtBP mediates transcriptional repression by Knirps, Kruppel and Snail in the Drosophila embryo.* Embo J, 1998. **17**(23): p. 7009-20.

75. Bianchi-Frias, D., et al., *Hairy transcriptional repression targets and cofactor recruitment in Drosophila.* PLoS Biol, 2004. **2**(7): p. E178.

76. Takata, T. and F. Ishikawa, *Human Sir2-related protein SIRT1 associates with the bHLH repressors HES1 and HEY2 and is involved in HES1- and HEY2-mediated transcriptional repression.* Biochem Biophys Res Commun, 2003. **301**(1): p. 250-7.

77. Shore, D., M. Squire, and K.A. Nasmyth, *Characterization of two genes required for the position-effect control of yeast mating-type genes.* Embo J, 1984. **3**(12): p. 2817-23.

78. Moazed, D., *Common themes in mechanisms of gene silencing.* Mol Cell, 2001. **8**(3): p. 489-98.

79. Rusche, L.N., A.L. Kirchmaier, and J. Rine, *Ordered nucleation and spreading of silenced chromatin in Saccharomyces cerevisiae.* Mol Biol Cell, 2002. **13**(7): p. 2207-22.

80. Moazed, D., et al., *A model for step-wise assembly of heterochromatin in yeast.* Novartis Found Symp, 2004. **259**: p. 48-56; discussion 56-62, 163-9.

81. Jennings, B.H., et al., *Molecular recognition of transcriptional repressor motifs by the WD domain of the Groucho/TLE corepressor.* Mol Cell, 2006. **22**(5): p. 645-55.

82. Copley, R.R., *The EH1 motif in metazoan transcription factors.* BMC Genomics, 2005. **6**: p. 169.

83. Neer, E.J., et al., *The ancient regulatory-protein family of WD-repeat proteins.* Nature, 1994. **371**(6495): p. 297-300.

84. Chen, G., et al., *A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development.* Genes Dev, 1999. **13**(17): p. 2218-30.

85. Flores-Saaib, R.D. and A.J. Courey, *Analysis of Groucho-histone interactions suggests mechanistic similarities between Groucho- and Tup1-mediated repression.* Nucleic Acids Res, 2000. **28**(21): p. 4189-96.

86. Choi, C.Y., et al., *Phosphorylation by the DHIPK2 protein kinase modulates the corepressor activity of Groucho.* J Biol Chem, 2005. **280**(22): p. 21427-36.

87. Nuthall, H.N., K. Joachim, and S. Stifani, *Phosphorylation of serine 239 of Groucho/TLE1 by protein kinase CK2 is important for inhibition of neuronal differentiation.* Mol Cell Biol, 2004. **24**(19): p. 8395-407.

88. Van Doren, M., H.M. Ellis, and J.W. Posakony, *The Drosophila extramacrochaetae protein antagonizes sequence-specific DNA binding by daughterless/achaete-scute protein complexes.* Development, 1991. **113**(1): p. 245-55.

89. Akazawa, C., et al., *Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system.* J Biol Chem, 1992. **267**(30): p. 21879-85.

90. Sun, J., et al., *Regulation of myogenic terminal differentiation by the hairy-related transcription factor CHF2.* J Biol Chem, 2001. **276**(21): p. 18591-6.

91. Chin, M.T., et al., *Cardiovascular basic helix loop helix factor 1, a novel transcriptional repressor expressed preferentially in the developing and adult cardiovascular system.* J Biol Chem, 2000. **275**(9): p. 6381-7.

92. Ghosh, B. and S.D. Leach, *Interactions between hairy/enhancer of split-related proteins and the pancreatic transcription factor Ptf1-p48 modulate function of the PTF1 transcriptional complex.* Biochem J, 2006. **393**(Pt 3): p. 679-85.

93. Jan, Y.N. and L.Y. Jan, *Functional gene cassettes in development.* Proc Natl Acad Sci U S A, 1993. **90**(18): p. 8305-7.

94. Hartenstein, V. and J.W. Posakony, *Development of adult sensilla on the wing and notum of Drosophila melanogaster.* Development, 1989. **107**(2): p. 389-405.

95. Campos-Ortega, J.A., *Genetic mechanisms of early neurogenesis in Drosophila melanogaster.* Mol Neurobiol, 1995. **10**(2-3): p. 75-89.

96. Jarman, A.P., et al., *Role of the proneural gene, atonal, in formation of Drosophila chordotonal organs and photoreceptors.* Development, 1995. **121**(7): p. 2019-30.

97. Caudy, M., et al., *daughterless, a Drosophila gene essential for both neurogenesis and sex determination, has sequence similarities to myc and the achaete-scute complex.* Cell, 1988. **55**(6): p. 1061-7.

98. Rushlow, C.A., et al., *The Drosophila hairy protein acts in both segmentation and bristle patterning and shows homology to N-myc.* Embo J, 1989. **8**(10): p. 3095-103.

99. Nakao, K. and J.A. Campos-Ortega, *Persistent expression of genes of the enhancer of split complex suppresses neural development in Drosophila.* Neuron, 1996. **16**(2): p. 275-86.

100. Bier, E., et al., *deadpan, an essential pan-neural gene in Drosophila, encodes a helix-loop-helix protein similar to the hairy gene product.* Genes Dev, 1992. **6**(11): p. 2137-51.

101. Cubas, P., et al., *Proneural clusters of achaete-scute expression and the generation of sensory organs in the Drosophila imaginal wing disc.* Genes Dev, 1991. **5**(6): p. 996-1008.

102. Simpson, P., *Lateral inhibition and the development of the sensory bristles of the adult peripheral nervous system of Drosophila.* Development, 1990. **109**(3): p. 509-19.

103. Tomlinson, A. and D.F. Ready, *Neuronal Differentiation in the Drosophila ommatidium.* Dev. Biol., 1987. **120**: p. 366-376.

104. Greenwood, S. and G. Struhl, *Progression of the morphogenetic furrow in the Drosophila eye: the roles of Hedgehog, Decapentaplegic and the Raf pathway.* Development, 1999. **126**(24): p. 5795-808.

105. Baylies, M.K. and M. Bate, *twist: a myogenic switch in Drosophila.* Science, 1996. **272**(5267): p. 1481-4.

106. Castanon, I. and M.K. Baylies, *A Twist in fate: evolutionary comparison of Twist structure and function.* Gene, 2002. **287**(1-2): p. 11-22.

107. Orenic, T.V., et al., *The spatial organization of epidermal structures: hairy establishes the geometrical pattern of Drosophila leg bristles by delimiting the domains of achaete expression.* Development, 1993. **118**(1): p. 9-20.

108. Tapanes-Castillo, A. and M.K. Baylies, *Notch signaling patterns Drosophila mesodermal segments by regulating the bHLH transcription factor twist.* Development, 2004. **131**(10): p. 2359-72.

109. Carmena, A., M. Bate, and F. Jimenez, *Lethal of scute, a proneural gene, participates in the specification of muscle progenitors during Drosophila embryogenesis.* Genes Dev, 1995. **9**(19): p. 2373-83.

110. Giebel, B., *The notch signaling pathway is required to specify muscle progenitor cells in Drosophila.* Mech Dev, 1999. **86**(1-2): p. 137-45.

111. Myat, M.M., *Making tubes in the Drosophila embryo.* Dev Dyn, 2005. **232**(3): p. 617-32.

112. Hoch, M., et al., *Sequential fates in a single cell are established by the neurogenic cascade in the Malpighian tubules of Drosophila.* Development, 1994. **120**(12): p. 3439-50.

113. Parkhurst, S.M., D. Bopp, and D. Ish-Horowicz, *X:A ratio, the primary sex-determining signal in Drosophila, is transduced by helix-loop-helix proteins.* Cell, 1990. **63**(6): p. 1179-91.

114. Myat, M.M. and D.J. Andrew, *Epithelial tube morphology is determined by the polarized growth and delivery of apical membrane.* Cell, 2002. **111**(6): p. 879-91.

115. Ohtsuka, T., et al., *Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation.* Embo J, 1999. **18**(8): p. 2196-207.

116. Ishibashi, M., et al., *Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects.* Genes Dev, 1995. **9**(24): p. 3136-48.

117. Cau, E., et al., *Hes genes regulate sequential stages of neurogenesis in the olfactory epithelium.* Development, 2000. **127**(11): p. 2323-32.

118. Takke, C., et al., *her4, a zebrafish homologue of the Drosophila neurogenic gene E(spl), is a target of NOTCH signalling.* Development, 1999. **126**(9): p. 1811-21.

119. Schneider, M.L., D.L. Turner, and M.L. Vetter, *Notch signaling can inhibit Xath5 function in the neural plate and developing retina.* Mol Cell Neurosci, 2001. **18**(5): p. 458-72.

120. Hojo, M., et al., *Glial cell fate specification modulated by the bHLH gene Hes5 in mouse retina.* Development, 2000. **127**(12): p. 2515-22.

121. Furukawa, T., et al., *rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells.* Neuron, 2000. **26**(2): p. 383-94.

122. Satow, T., et al., *The basic helix-loop-helix gene hesr2 promotes gliogenesis in mouse retina.* J Neurosci, 2001. **21**(4): p. 1265-73.

123. Bae, S., et al., *The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation.* Development, 2000. **127**(13): p. 2933-43.

124. Kageyama, R., T. Ohtsuka, and T. Kobayashi, *The Hes gene family: repressors and oscillators that orchestrate embryogenesis.* Development, 2007. **134**(7): p. 1243-51.

125. Ohtsuka, T., et al., *Roles of the basic helix-loop-helix genes Hes1 and Hes5 in expansion of neural stem cells of the developing brain.* J Biol Chem, 2001. **276**(32): p. 30467-74.

126. Hatakeyama, J., et al., *Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation.* Development, 2004. **131**(22): p. 5539-50.

127. Baek, J.H., et al., *Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system.* Development, 2006. **133**(13): p. 2467-76.

128. Lamont, R.E. and S. Childs, *MAPping out arteries and veins.* Sci STKE, 2006. **2006**(355): p. pe39.

129. Zhong, T.P., et al., *gridlock, an HLH gene required for assembly of the aorta in zebrafish.* Science, 2000. **287**(5459): p. 1820-4.

130. Kokubo, H., et al., *Mouse hesr1 and hesr2 genes are redundantly required to mediate Notch signaling in the developing cardiovascular system.* Dev Biol, 2005. **278**(2): p. 301-9.

131. Fischer, A., et al., *The Notch target genes Hey1 and Hey2 are required for embryonic vascular development.* Genes Dev, 2004. **18**(8): p. 901-11.

132. Henderson, A.M., et al., *The basic helix-loop-helix transcription factor HESR1 regulates endothelial cell tube formation.* J Biol Chem, 2001. **276**(9): p. 6169-76.

133. Lawson, N.D., et al., *Notch signaling is required for arterial-venous differentiation during embryonic vascular development.* Development, 2001. **128**(19): p. 3675-83.

134. Rutenberg, J.B., et al., *Developmental patterning of the cardiac atrioventricular canal by Notch and Hairy-related transcription factors.* Development, 2006. **133**(21): p. 4381-90.

135. Kokubo, H., et al., *Hesr1 and Hesr2 regulate atrioventricular boundary formation in the developing heart through the repression of Tbx2.* Development, 2007. **134**(4): p. 747-55.

136. Fischer, A., et al., *Combined loss of Hey1 and HeyL causes congenital heart defects because of impaired epithelial to mesenchymal transition.* Circ Res, 2007. **100**(6): p. 856-63.

137. Garg, V., et al., *Mutations in NOTCH1 cause aortic valve disease.* Nature, 2005. **437**(7056): p. 270-4.

138. Palmeirim, I., et al., *Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis.* Cell, 1997. **91**(5): p. 639-48.

139. Lawrence, P.A. and P. Johnston, *Pattern formation in the Drosophila embryo: allocation of cells to parasegments by even-skipped and fushi tarazu.* Development, 1989. **105**(4): p. 761-7.

140. Giudicelli, F. and J. Lewis, *The vertebrate segmentation clock.* Curr Opin Genet Dev, 2004. **14**(4): p. 407-14.

141. Jouve, C., et al., *Notch signalling is required for cyclic expression of the hairy-like gene HES1 in the presomitic mesoderm.* Development, 2000. **127**(7): p. 1421-9.

142. Leimeister, C., et al., *Oscillating expression of c-Hey2 in the presomitic mesoderm suggests that the segmentation clock may use combinatorial signaling through multiple interacting bHLH factors.* Dev Biol, 2000. **227**(1): p. 91-103.

143. Bessho, Y., et al., *Dynamic expression and essential functions of Hes7 in somite segmentation.* Genes Dev, 2001. **15**(20): p. 2642-7.

144. Holley, S.A., R. Geisler, and C. Nusslein-Volhard, *Control of her1 expression during zebrafish somitogenesis by a delta-dependent oscillator and an independent wave-front activity.* Genes Dev, 2000. **14**(13): p. 1678-90.

145. Bessho, Y. and R. Kageyama, *Oscillations, clocks and segmentation.* Curr Opin Genet Dev, 2003. **13**(4): p. 379-84.

146. Hirata, H., et al., *Instability of Hes7 protein is crucial for the somite segmentation clock.* Nat Genet, 2004. **36**(7): p. 750-4.

147. Rida, P.C., N. Le Minh, and Y.J. Jiang, *A Notch feeling of somite segmentation and beyond.* Dev Biol, 2004. **265**(1): p. 2-22.

148. Umbhauer, M., J.C. Boucaut, and D.L. Shi, *Repression of XMyoD expression and myogenesis by Xhairy-1 in Xenopus early embryo.* Mech Dev, 2001. **109**(1): p. 61-8.

149. Gao, X., et al., *HES6 acts as a transcriptional repressor in myoblasts and can induce the myogenic differentiation program.* J Cell Biol, 2001. **154**(6): p. 1161-71.

150. Kuroda, K., et al., *Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis.* J Biol Chem, 1999. **274**(11): p. 7238-44.

151. Hirsinger, E., et al., *Notch signalling acts in postmitotic avian myogenic cells to control MyoD activation.* Development, 2001. **128**(1): p. 107-16.

152. Kopan, R., J.S. Nye, and H. Weintraub, *The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD.* Development, 1994. **120**(9): p. 2385-96.

153. Nye, J.S., R. Kopan, and R. Axel, *An activated Notch suppresses neurogenesis and myogenesis but not gliogenesis in mammalian cells.* Development, 1994. **120**(9): p. 2421-30.

154. Tomita, K., et al., *The bHLH gene Hes1 is essential for expansion of early T cell precursors.* Genes Dev, 1999. **13**(9): p. 1203-10.

155. Kim, H.K. and G. Siu, *The notch pathway intermediate HES-1 silences CD4 gene expression.* Mol Cell Biol, 1998. **18**(12): p. 7166-75.

156. Siu, G., et al., *A transcriptional silencer controls the developmental expression of the CD4 gene.* Embo J, 1994. **13**(15): p. 3570-9.

157. Murata, K., et al., *Hes1 directly controls cell proliferation through the transcriptional repression of p27Kip1.* Mol Cell Biol, 2005. **25**(10): p. 4262-71.

158. Sumazaki, R., et al., *Conversion of biliary system to pancreatic tissue in Hes1-deficient mice.* Nat Genet, 2004. **36**(1): p. 83-7.

159. Fukuda, A., et al., *Ectopic pancreas formation in Hes1 -knockout mice reveals plasticity of endodermal progenitors of the gut, bile duct, and pancreas.* J Clin Invest, 2006. **116**(6): p. 1484-93.

160. Jensen, J., et al., *Control of endodermal endocrine development by Hes-1.* Nat Genet, 2000. **24**(1): p. 36-44.

161. Yang, Q., et al., *Requirement of Math1 for secretory cell lineage commitment in the mouse intestine.* Science, 2001. **294**(5549): p. 2155-8.

162. Fre, S., et al., *Notch signals control the fate of immature progenitor cells in the intestine.* Nature, 2005. **435**(7044): p. 964-8.

163. Suzuki, K., et al., *Hes1-deficient mice show precocious differentiation of Paneth cells in the small intestine.* Biochem Biophys Res Commun, 2005. **328**(1): p. 348-52.

164. Stanger, B.Z., et al., *Direct regulation of intestinal fate by Notch.* Proc Natl Acad Sci U S A, 2005. **102**(35): p. 12443-8.

165. Kodama, Y., et al., *The role of notch signaling in the development of intrahepatic bile ducts.* Gastroenterology, 2004. **127**(6): p. 1775-86.

166. Axelson, H., *The Notch signaling cascade in neuroblastoma: role of the basic helix-loop-helix proteins HASH-1 and HES-1.* Cancer Lett, 2004. **204**(2): p. 171-8.

167. Kunnimalaiyaan, M., K. Traeger, and H. Chen, *Conservation of the Notch1 signaling pathway in gastrointestinal carcinoid cells.* Am J Physiol Gastrointest Liver Physiol, 2005. **289**(4): p. G636-42.

168. Kunnimalaiyaan, M., et al., *Hairy Enhancer of Split-1 (HES-1), a Notch1 effector, inhibits the growth of carcinoid tumor cells.* Surgery, 2005. **138**(6): p. 1137-42; discussion 1142.

169. Sriuranpong, V., et al., *Notch signaling induces cell cycle arrest in small cell lung cancer cells.* Cancer Res, 2001. **61**(7): p. 3200-5.

170. Chen, H., et al., *Conservation of the Drosophila lateral inhibition pathway in human lung cancer: a hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression.* Proc Natl Acad Sci U S A, 1997. **94**(10): p. 5355-60.

171. Ellisen, L.W., et al., *TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms.* Cell, 1991. **66**(4): p. 649-61.

172. Shimizu, D., et al., *Detection of NOTCH1 mutations in adult T-cell leukemia/lymphoma and peripheral T-cell lymphoma.* Int J Hematol, 2007. **85**(3): p. 212-8.

173. Lee, J.S., et al., *Murine leukemia provirus-mediated activation of the Notch1 gene leads to induction of HES-1 in a mouse T lymphoma cell line, DL-3.* FEBS Lett, 1999. **455**(3): p. 276-80.

174. Dumortier, A., et al., *Notch activation is an early and critical event during T-Cell leukemogenesis in Ikaros-deficient mice.* Mol Cell Biol, 2006. **26**(1): p. 209-20.

175. van Es, J.H., et al., *Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells.* Nature, 2005. **435**(7044): p. 959-63.

176. Lohr, D., P. Venkov, and J. Zlatanova, *Transcriptional regulation in the yeast GAL gene family: a complex genetic network.* Faseb J, 1995. **9**(9): p. 777-87.

177. Duffy, J.B., *GAL4 system in Drosophila: a fly geneticist's Swiss army knife.* Genesis, 2002. **34**(1-2): p. 1-15.

178. Marmorstein, R., et al., *DNA recognition by GAL4: structure of a protein-DNA complex.* Nature, 1992. **356**(6368): p. 408-14.

179. Giniger, E. and M. Ptashne, *Cooperative DNA binding of the yeast transcriptional activator GAL4.* Proc Natl Acad Sci U S A, 1988. **85**(2): p. 382-6.

180. Traven, A., B. Jelicic, and M. Sopta, *Yeast Gal4: a transcriptional paradigm revisited.* EMBO Rep, 2006. **7**(5): p. 496-9.

181. Wu, Y., R.J. Reece, and M. Ptashne, *Quantitation of putative activator-target affinities predicts transcriptional activating potentials.* Embo J, 1996. **15**(15): p. 3951-63.

182. Melcher, K. and S.A. Johnston, *GAL4 interacts with TATA-binding protein and coactivators.* Mol Cell Biol, 1995. **15**(5): p. 2839-48.

183. Ansari, A.Z., R.J. Reece, and M. Ptashne, *A transcriptional activating region with two contrasting modes of protein interaction.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13543-8.

184. Koh, S.S., et al., *An activator target in the RNA polymerase II holoenzyme.* Mol Cell, 1998. **1**(6): p. 895-904.

185. Jeong, C.J., et al., *Evidence that Gal11 protein is a target of the Gal4 activation domain in the mediator.* Biochemistry, 2001. **40**(31): p. 9421-7.

186. Bhaumik, S.R. and M.R. Green, *SAGA is an essential in vivo target of the yeast acidic activator Gal4p.* Genes Dev, 2001. **15**(15): p. 1935-45.

187. Ferreira, M.E., et al., *Mechanism of transcription factor recruitment by acidic activators.* J Biol Chem, 2005. **280**(23): p. 21779-84.

188. Chang, C., et al., *The Gal4 activation domain binds Sug2 protein, a proteasome component, in vivo and in vitro.* J Biol Chem, 2001. **276**(33): p. 30956-63.

189. Gonzalez, F., et al., *Recruitment of a 19S proteasome subcomplex to an activated promoter.* Science, 2002. **296**(5567): p. 548-50.

190. Lum, J.K. and A.K. Mapp, *Artificial transcriptional activation domains.* Chembiochem, 2005. **6**(8): p. 1311-5.

191. Hollenberg, S.M. and R.M. Evans, *Multiple and cooperative trans-activation domains of the human glucocorticoid receptor.* Cell, 1988. **55**(5): p. 899-906.

192. Neely, K.E., et al., *Activation domain-mediated targeting of the SWI/SNF complex to promoters stimulates transcription from nucleosome arrays.* Mol Cell, 1999. **4**(4): p. 649-55.

193. Schaeffer, V., et al., *Bicoid functions without its TATA-binding protein-associated factor interaction domains.* Proc Natl Acad Sci U S A, 1999. **96**(8): p. 4461-6.

194. Driever, W., et al., *Rescue of bicoid mutant Drosophila embryos by bicoid fusion proteins containing heterologous activating sequences.* Nature, 1989. **342**(6246): p. 149-54.

195. Grant, P.A., et al., *Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex.* Genes Dev, 1997. **11**(13): p. 1640-50.

196. Sterner, D.E., et al., *Functional organization of the yeast SAGA complex: distinct components involved in structural integrity, nucleosome acetylation, and TATA-binding protein interaction.* Mol Cell Biol, 1999. **19**(1): p. 86-98.

197. Candau, R., et al., *Histone acetyltransferase activity and interaction with ADA2 are critical for GCN5 function in vivo.* Embo J, 1997. **16**(3): p. 555-65.

198. Barlev, N.A., et al., *Characterization of physical interactions of the putative transcriptional adaptor, ADA2, with acidic activation domains and TATA-binding protein.* J Biol Chem, 1995. **270**(33): p. 19337-44.

199. Saleh, A., et al., *Identification of native complexes containing the yeast coactivator/repressor proteins NGG1/ADA3 and ADA2.* J Biol Chem, 1997. **272**(9): p. 5571-8.

200. Grant, P.A., et al., *The ATM-related cofactor Tra1 is a component of the purified SAGA complex.* Mol Cell, 1998. **2**(6): p. 863-7.

201. Allard, S., et al., *NuA4, an essential transcription adaptor/histone H4 acetyltransferase complex containing Esa1p and the ATM-related cofactor Tra1p.* Embo J, 1999. **18**(18): p. 5108-19.

202. Henry, K.W., et al., *Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8.* Genes Dev, 2003. **17**(21): p. 2648-63.

203. Bryant, G.O. and M. Ptashne, *Independent recruitment in vivo by Gal4 of two complexes required for transcription.* Mol Cell, 2003. **11**(5): p. 1301-9.

204. Bhaumik, S.R., et al., *In vivo target of a transcriptional activator revealed by fluorescence resonance energy transfer.* Genes Dev, 2004. **18**(3): p. 333-43.

205. Barbaric, S., H. Reinke, and W. Horz, *Multiple mechanistically distinct functions of SAGA at the PHO5 promoter.* Mol Cell Biol, 2003. **23**(10): p. 3468-76.

206. Gregory, P.D., et al., *Chromatin remodelling at the PHO8 promoter requires SWI-SNF and SAGA at a step subsequent to activator binding.* Embo J, 1999. **18**(22): p. 6407-14.

207. Ornaghi, P., et al., *The bromodomain of Gcn5p interacts in vitro with specific residues in the N terminus of histone H4.* J Mol Biol, 1999. **287**(1): p. 1-7.

208. Kusch, T., et al., *Two Drosophila Ada2 homologues function in different multiprotein complexes.* Mol Cell Biol, 2003. **23**(9): p. 3305-19.

209. Peterson, C.L. and I. Herskowitz, *Characterization of the yeast SWI1, SWI2, and SWI3 genes, which encode a global activator of transcription.* Cell, 1992. **68**(3): p. 573-83.

210. Gutierrez, J.L., et al., *Activation domains drive nucleosome eviction by SWI/SNF.* Embo J, 2007. **26**(3): p. 730-40.

211. Mitra, D., et al., *SWI/SNF binding to the HO promoter requires histone acetylation and stimulates TATA-binding protein recruitment.* Mol Cell Biol, 2006. **26**(11): p. 4095-110.

212. Lohr, D., *Nucleosome transactions on the promoters of the yeast GAL and PHO genes.* J Biol Chem, 1997. **272**(43): p. 26795-8.

213. Dhasarathy, A. and M.P. Kladde, *Promoter occupancy is a major determinant of chromatin remodeling enzyme requirements.* Mol Cell Biol, 2005. **25**(7): p. 2698-707.

214. Sudarsanam, P. and F. Winston, *The Swi/Snf family nucleosome-remodeling complexes and transcriptional control.* Trends Genet, 2000. **16**(8): p. 345-51.

215. Kao, C.F., et al., *Rad6 plays a role in transcriptional activation through ubiquitylation of histone H2B.* Genes Dev, 2004. **18**(2): p. 184-95.

216. Dover, J., et al., *Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6.* J Biol Chem, 2002. **277**(32): p. 28368-71.

217. Briggs, S.D., et al., *Gene silencing: trans-histone regulatory pathway in chromatin.* Nature, 2002. **418**(6897): p. 498.

218. Xiao, T., et al., *Histone H2B ubiquitylation is associated with elongating RNA polymerase II.* Mol Cell Biol, 2005. **25**(2): p. 637-51.

219. Wood, A., et al., *The Paf1 complex is essential for histone monoubiquitination by the Rad6-Bre1 complex, which signals for histone methylation by COMPASS and Dot1p.* J Biol Chem, 2003. **278**(37): p. 34739-42.

220. Krogan, N.J., et al., *The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation.* Mol Cell, 2003. **11**(3): p. 721-9.

221. Shahbazian, M.D., K. Zhang, and M. Grunstein, *Histone H2B ubiquitylation controls processive methylation but not monomethylation by Dot1 and Set1.* Mol Cell, 2005. **19**(2): p. 271-7.

222. Wood, A., et al., *The Bur1/Bur2 complex is required for histone H2B monoubiquitination by Rad6/Bre1 and histone methylation by COMPASS.* Mol Cell, 2005. **20**(4): p. 589-99.

223. Keogh, M.C., V. Podolny, and S. Buratowski, *Bur1 kinase is required for efficient transcription elongation by RNA polymerase II.* Mol Cell Biol, 2003. **23**(19): p. 7005-18.

224. Pray-Grant, M.G., et al., *Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation.* Nature, 2005. **433**(7024): p. 434-8.

225. Santos-Rosa, H., et al., *Methylation of histone H3 K4 mediates association of the Isw1p ATPase with chromatin.* Mol Cell, 2003. **12**(5): p. 1325-32.

226. Ingham, P., *Abdominal gene organization.* Nature, 1985. **313**(5998): p. 98-9.

227. Chen, G. and A.J. Courey, *Groucho/TLE family proteins and transcriptional repression.* Gene, 2000. **249**(1-2): p. 1-16.

228. Wharton, K.A. and S.T. Crews, *CNS midline enhancers of the Drosophila slit and Toll genes.* Mech. Dev., 1993. **40**(3): p. 141-154.

229. Kulkarni, M.M. and D.N. Arnosti, *cis-regulatory logic of short-range transcriptional repression in Drosophila melanogaster.* Mol Cell Biol, 2005. **25**(9): p. 3411-20.

230. Kulkarni, M.M., *The function and design of cis-acting enhancer elements regulated by short-range transcriptional repressors: grammar studies from Drosophila melanogaster.* Dissertation for the degree of PhD. Michigan State University, 2003.

231. Small, S., A. Blair, and M. Levine, *Regulation of even-skipped stripe 2 in the Drosophila embryo.* Embo J, 1992. **11**(11): p. 4047-57.

232. Pirrotta, V., *Vectors for P-mediated transformation in Drosophila.* Biotechnology, 1988. **10**: p. 437-56.

233. Stebbins, J.L. and S.J. Triezenberg, *Identification, mutational analysis, and coactivator requirements of two distinct transcriptional activation domains of the Saccharomyces cerevisiae Hap4 protein.* Eukaryot Cell, 2004. **3**(2): p. 339-47.

234. Larschan, E. and F. Winston, *The S. cerevisiae SAGA complex functions in vivo as a coactivator for transcriptional activation by Gal4.* Genes Dev, 2001. **15**(15): p. 1946-56.

235. Nalley, K., S.A. Johnston, and T. Kodadek, *Proteolytic turnover of the Gal4 transcription factor is not required for function in vivo.* Nature, 2006. **442**(7106): p. 1054-7.

236. Lee, M., S. Chatterjee, and K. Struhl, *Genetic analysis of the role of Pol II holoenzyme components in repression by the Cyc8-Tup1 corepressor in yeast.* Genetics, 2000. **155**(4): p. 1535-42.

237. Green, S.R. and A.D. Johnson, *Genome-wide analysis of the functions of a conserved surface on the corepressor Tup1.* Mol Biol Cell, 2005. **16**(6): p. 2605-13.

238. Hallberg, M., et al., *Functional and physical interactions within the middle domain of the yeast mediator.* Mol Genet Genomics, 2006. **276**(2): p. 197-210.

239. Zeitlinger, J., et al., *Whole-genome ChIP-chip analysis of Dorsal, Twist, and Snail suggests integration of diverse patterning processes in the Drosophila embryo.* Genes Dev, 2007. **21**(4): p. 385-90.

240. Small, S., D.N. Arnosti, and M. Levine, *Spacing ensures autonomous expression of different stripe enhancers in the even-skipped promoter.* Development, 1993. **119**(3): p. 762-72.

241. Jiang, J., et al., *Conversion of a dorsal-dependent silencer into an enhancer: evidence for dorsal corepressors.* Embo J, 1993. **12**(8): p. 3201-9.

242. Kurdistani, S.K. and M. Grunstein, *In vivo protein-protein and protein-DNA crosslinking for genomewide binding microarray.* Methods, 2003. **31**(1): p. 90-5.

243. Zeng, P.Y., et al., *In vivo dual cross-linking for identification of indirect DNA-associated proteins by chromatin immunoprecipitation.* Biotechniques, 2006. **41**(6): p. 694, 696, 698.

244. Song, H., et al., *Groucho oligomerization is required for repression in vivo.* Mol Cell Biol, 2004. **24**(10): p. 4341-50.

245. Davie, J.K., et al., *Tup1-Ssn6 interacts with multiple class I histone deacetylases in vivo.* J Biol Chem, 2003. **278**(50): p. 50158-62.

246. Huang, L., W. Zhang, and S.Y. Roth, *Amino termini of histones H3 and H4 are required for a1-alpha2 repression in yeast.* Mol Cell Biol, 1997. **17**(11): p. 6555-62.

247. Watson, A.D., et al., *Ssn6-Tup1 interacts with class I histone deacetylases required for repression.* Genes Dev, 2000. **14**(21): p. 2737-44.

248. Tripic, T., et al., *The Set2 methyltransferase associates with Ssn6 yet Tup1-Ssn6 repression is independent of histone methylation.* Biochem Biophys Res Commun, 2006. **339**(3): p. 905-14.

249. Ducker, C.E. and R.T. Simpson, *The organized chromatin domain of the repressed yeast a cell-specific gene STE6 contains two molecules of the corepressor Tup1p per nucleosome.* Embo J, 2000. **19**(3): p. 400-9.

250. Wu, J., et al., *TUP1 utilizes histone H3/H2B-specific HDA1 deacetylase to repress gene activity in yeast.* Mol Cell, 2001. **7**(1): p. 117-26.

251. Davie, J.K., R.J. Trumbly, and S.Y. Dent, *Histone-dependent association of Tup1-Ssn6 with repressed genes in vivo.* Mol Cell Biol, 2002. **22**(3): p. 693-703.

252. Muller, J. and J.A. Kassis, *Polycomb response elements and targeting of Polycomb group proteins in Drosophila.* Curr Opin Genet Dev, 2006. **16**(5): p. 476-84.

253. Schwartz, Y.B. and V. Pirrotta, *Polycomb silencing mechanisms and the management of genomic programmes.* Nat Rev Genet, 2007. **8**(1): p. 9-22.

254. Sekinger, E.A. and D.S. Gross, *SIR repression of a yeast heat shock gene: UAS and TATA footprints persist within heterochromatin.* Embo J, 1999. **18**(24): p. 7041-55.

255. Sekinger, E.A. and D.S. Gross, *Silenced chromatin is permissive to activator binding and PIC recruitment.* Cell, 2001. **105**(3): p. 403-14.

256. King, I.F., N.J. Francis, and R.E. Kingston, *Native and recombinant polycomb group complexes establish a selective block to template accessibility to repress transcription in vitro.* Mol Cell Biol, 2002. **22**(22): p. 7919-28.

257. Gilmour, D.S. and J.T. Lis, *RNA polymerase II interacts with the promoter region of the noninduced hsp70 gene in Drosophila melanogaster cells.* Mol Cell Biol, 1986. **6**(11): p. 3984-9.

258. Wang, X., et al., *Transcription elongation controls cell fate specification in the Drosophila embryo.* Genes Dev, 2007. **21**(9): p. 1031-6.

259. Beuchle, D., G. Struhl, and J. Muller, *Polycomb group proteins and heritable silencing of Drosophila Hox genes.* Development, 2001. **128**(6): p. 993-1004.

260. Fischer, A. and M. Gessler, *Delta-Notch--and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors.* Nucleic Acids Res, 2007.

261. Iso, T., L. Kedes, and Y. Hamamori, *HES and HERP families: multiple effectors of the Notch signaling pathway.* J Cell Physiol, 2003. **194**(3): p. 237-55.

262. Joshi, M., et al., *Delta and Hairy establish a periodic prepattern that positions sensory bristles in Drosophila legs.* Dev Biol, 2006. **293**(1): p. 64-76.

263. Geling, A., et al., *Her5 acts as a prepattern factor that blocks neurogenin1 and coe2 expression upstream of Notch to inhibit neurogenesis at the midbrain-hindbrain boundary.* Development, 2004. **131**(9): p. 1993-2006.

264. Stathopoulos, A. and M. Levine, *Linear signaling in the Toll-Dorsal pathway of Drosophila: activated Pelle kinase specifies all threshold outputs of gene expression while the bHLH protein Twist specifies a subset.* Development, 2002. **129**(14): p. 3411-9.

265. Aronson, B.D., et al., *Groucho-dependent and -independent repression activities of Runt domain proteins.* Mol Cell Biol, 1997. **17**(9): p. 5581-7.

266. Edmondson, D.G., M.M. Smith, and S.Y. Roth, *Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4.* Genes Dev, 1996. **10**(10): p. 1247-59.

267. Euskirchen, G.M., et al., *Mapping of transcription factor binding regions in mammalian cells by ChIP: comparison of array- and sequencing-based technologies.* Genome Res, 2007. **17**(6): p. 898-909.

268. Roseman, R.R., V. Pirrotta, and P.K. Geyer, *The su(Hw) protein insulates expression of the Drosophila melanogaster white gene from chromosomal position-effects.* Embo J, 1993. **12**(2): p. 435-42.

269. Pikaart, M.J., F. Recillas-Targa, and G. Felsenfeld, *Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators.* Genes Dev, 1998. **12**(18): p. 2852-62.

270. Liaw, H. and A.J. Lustig, *Sir3 C-terminal domain involvement in the initiation and spreading of heterochromatin.* Mol Cell Biol, 2006. **26**(20): p. 7616-31.

271. Hoppe, G.J., et al., *Steps in assembly of silent chromatin in yeast: Sir3-independent binding of a Sir2/Sir4 complex to silencers and role for Sir2-dependent deacetylation.* Mol Cell Biol, 2002. **22**(12): p. 4167-80.

272. Malave, T.M. and S.Y. Dent, *Transcriptional repression by Tup1-Ssn6.* Biochem Cell Biol, 2006. **84**(4): p. 437-43.

273. Lee, J.S. and A. Shilatifard, *A site to remember: H3K36 methylation a mark for histone deacetylation.* Mutat Res, 2007. **618**(1-2): p. 130-4.

274. Joshi, A.A. and K. Struhl, *Eaf3 chromodomain interaction with methylated H3-K36 links histone deacetylation to Pol II elongation.* Mol Cell, 2005. **20**(6): p. 971-8.

275. Li, B., et al., *Combined action of PHD and chromo domains directs the Rpd3S HDAC to transcribed chromatin.* Science, 2007. **316**(5827): p. 1050-4.

276. Krogan, N.J., et al., *A Snf2 family ATPase complex required for recruitment of the histone H2A variant Htz1.* Mol Cell, 2003. **12**(6): p. 1565-76.

277. Mizuguchi, G., et al., *ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex.* Science, 2004. **303**(5656): p. 343-8.

278. Meneghini, M.D., M. Wu, and H.D. Madhani, *Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin.* Cell, 2003. **112**(5): p. 725-36.

279. Raisner, R.M., et al., *Histone variant H2A.Z marks the 5' ends of both active and inactive genes in euchromatin.* Cell, 2005. **123**(2): p. 233-48.

280. Zhang, H., D.N. Roberts, and B.R. Cairns, *Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss.* Cell, 2005. **123**(2): p. 219-31.

281. Gligoris, T., G. Thireos, and D. Tzamarias, *The Tup1 corepressor directs Htz1 deposition at a specific promoter nucleosome marking the GAL1 gene for rapid activation.* Mol Cell Biol, 2007. **27**(11): p. 4198-205.

282. Kobor, M.S., et al., *A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin.* PLoS Biol, 2004. **2**(5): p. E131.

283. Andrioli, L.P., et al., *Groucho-dependent repression by sloppy-paired 1 differentially positions anterior pair-rule stripes in the Drosophila embryo.* Dev Biol, 2004. **276**(2): p. 541-51.

284. Hasson, P., et al., *Brinker requires two corepressors for maximal and versatile repression in Dpp signalling.* Embo J, 2001. **20**(20): p. 5725-36.

285. Nagel, A.C., et al., *Hairless-mediated repression of notch target genes requires the combined activity of Groucho and CtBP corepressors.* Mol Cell Biol, 2005. **25**(23): p. 10433-41.

286. Shahbazian, M.D. and M. Grunstein, *Functions of site-specific histone acetylation and deacetylation.* Annu Rev Biochem, 2007. **76**: p. 75-100.

287. Suka, N., K. Luo, and M. Grunstein, *Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin.* Nat Genet, 2002. **32**(3): p. 378-83.

288. Kimura, A., T. Umehara, and M. Horikoshi, *Chromosomal gradient of histone acetylation established by Sas2p and Sir2p functions as a shield against gene silencing.* Nat Genet, 2002. **32**(3): p. 370-7.

289. Millar, C.B., S.K. Kurdistani, and M. Grunstein, *Acetylation of yeast histone H4 lysine 16: a switch for protein interactions in heterochromatin and euchromatin.* Cold Spring Harb Symp Quant Biol, 2004. **69**: p. 193-200.

290. Babiarz, J.E., J.E. Halley, and J. Rine, *Telomeric heterochromatin boundaries require NuA4-dependent acetylation of histone variant H2A.Z in Saccharomyces cerevisiae.* Genes Dev, 2006. **20**(6): p. 700-10.

291. Bi, X., et al., *Formation of boundaries of transcriptionally silent chromatin by nucleosome-excluding structures.* Mol Cell Biol, 2004. **24**(5): p. 2118-31.

292. Oki, M. and R.T. Kamakaka, *Barrier function at HMR.* Mol Cell, 2005. **19**(5): p. 707-16.

293. Tompa, R. and H.D. Madhani, *Histone H3 lysine 36 methylation antagonizes silencing in Saccharomyces cerevisiae independently of the Rpd3S histone deacetylase complex.* Genetics, 2007. **175**(2): p. 585-93.

294. Zinzen, R.P., et al., *Computational models for neurogenic gene expression in the Drosophila embryo.* Curr Biol, 2006. **16**(13): p. 1358-65.

295. Janssens, H., et al., *Quantitative and predictive model of transcriptional control of the Drosophila melanogaster even skipped gene.* Nat Genet, 2006. **38**(10): p. 1159-65.

296. Zinzen, R.P. and D. Papatsenko, *Enhancer responses to similarly distributed antagonistic gradients in development.* PLoS Comput Biol, 2007. **3**(5): p. e84.

297. Arnosti, D.N. and M.M. Kulkarni, *Transcriptional enhancers: Intelligent enhanceosomes or flexible billboards?* J Cell Biochem, 2005. **94**(5): p. 890-8.

298. Ludwig, M.Z., N.H. Patel, and M. Kreitman, *Functional analysis of eve stripe 2 enhancer evolution in Drosophila: rules governing conservation and change.* Development, 1998. **125**(5): p. 949-58.

299. Kulkarni, M.M. and D.N. Arnosti, *Information display by transcriptional enhancers.* Development, 2003. **130**(26): p. 6569-75.

300. Keller, S.A., et al., *dCtBP-dependent and -independent repression activities of the Drosophila Knirps protein.* Mol Cell Biol, 2000. **20**(19): p. 7247-58.