

Hairy Transcriptional Repression Targets and Cofactor Recruitment in *Drosophila*

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Members of the widely conserved Hairy/Enhancer of split family of basic Helix-Loop-Helix repressors are essential for proper *Drosophila* and vertebrate development and are misregulated in many cancers. While a major step forward in understanding the molecular mechanism(s) surrounding Hairy-mediated repression was made with the identification of Groucho, *Drosophila* C-terminal binding protein (dCtBP), and *Drosophila* silent information regulator 2 (dSir2) as Hairy transcriptional cofactors, the identity of Hairy target genes and the rules governing cofactor recruitment are relatively unknown. We have used the chromatin profiling method DamID to perform a global and systematic search for direct transcriptional targets for *Drosophila* Hairy and the genomic recruitment sites for three of its cofactors: Groucho, dCtBP, and dSir2. Each of the proteins was tethered to *Escherichia coli* DNA adenine methyltransferase, permitting methylation proximal to in vivo binding sites in both *Drosophila* Kc cells and early embryos. This approach identified 40 novel genomic targets for Hairy in Kc cells, as well as 155 loci recruiting Groucho, 107 loci recruiting dSir2, and wide genomic binding of dCtBP to 496 loci. We also adapted DamID profiling such that we could use tightly gated collections of embryos (2–6 h) and found 20 Hairy targets related to early embryogenesis. As expected of direct targets, all of the putative Hairy target genes tested show Hairy-dependent expression and have conserved consensus C-box-containing sequences that are directly bound by Hairy in vitro. The distribution of Hairy targets in both the Kc cell and embryo DamID experiments corresponds to Hairy binding sites in vivo on polytene chromosomes. Similarly, the distributions of loci recruiting each of Hairy's cofactors are detected as cofactor binding sites in vivo on polytene chromosomes. We have identified 59 putative transcriptional targets of Hairy. In addition to finding putative targets for Hairy in segmentation, we find groups of targets suggesting roles for Hairy in cell cycle, cell growth, and morphogenesis, processes that must be coordinately regulated with pattern formation. Examining the recruitment of Hairy's three characterized cofactors to their putative target genes revealed that cofactor recruitment is context-dependent. While Groucho is frequently considered to be the primary Hairy cofactor, we find here that it is associated with only a minority of Hairy targets. The majority of Hairy targets are associated with the presence of a combination of dCtBP and dSir2. Thus, the DamID chromatin profiling technique provides a systematic means of identifying transcriptional target genes and of obtaining a global view of cofactor recruitment requirements during development.

Introduction

Transcriptional repression is an important feature of developmental processes, where it is necessary for establishing intricate patterns of gene expression (reviewed in Herschbach and Johnson 1993; Gray and Levine 1996; Hanna-Rose and Hansen 1996; Courey and Jia 2001; Gaston and Jayaraman 2003). *Drosophila* embryogenesis is marked by the subdivision of the embryo into progressively more precise spatial domains, achieved through the coordinated functions of both transcriptional activators and repressors (maternal→gap→pair-rule→segment polarity; for review, see Lawrence 1992). One such developmental repressor is the pair-rule gene *hairy*, which sits at a key position in the segmentation gene hierarchy: it is one of the first genes to show the reiterated periodicity that is central to the establishment of proper embryonic body plan throughout metazoa (Ingham et al. 1985).

During segmentation, *hairy* behaves genetically as a negative regulator of a downstream (secondary) pair-rule gene, *fushi tarazu* (*ftz*; Carroll and Scott 1986; Howard and Ingham 1986). In addition to embryonic segmentation, Hairy also regulates several other developmental processes (cf. Brown et al. 1995; Davis and Turner 2001; Myat and Andrew 2002). For example, during larval development, Hairy is

required for proper peripheral nervous system development, where it is a negative regulator of the proneural basic Helix-Loop-Helix (bHLH) activator gene *achaete* (*ac*; Botas et al. 1982; Ohsako et al. 1994; Van Doren et al. 1994).

Hairy belongs to the evolutionarily conserved Hairy/Enhancer of split/Deadpan (HES) subclass of repressor bHLH proteins (Rushlow et al. 1989). These proteins function throughout development as dedicated transcriptional repressors of genes necessary for cell fate decisions in processes including segmentation, myogenesis, somitogenesis, sex determination, vasculogenesis, mesoderm formation, and neu-

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Abbreviations: *ac*, *achaete*; bHLH, basic Helix-Loop-Helix; CtBP, C-terminal binding protein; Dam, DNA adenine methyltransferase; dCtBP, *Drosophila* C-terminal binding protein; DGC, *Drosophila* Gene Collection; dSir2, *Drosophila* silent information regulator 2; *egh*, *egghead*; EMSA, electromobility shift assay; *ftz*, *fushi tarazu*; HES, Hairy/Enhancer of split/Deadpan; *hkb*, *huckebein*; NAD, nicotinamide adenine dinucleotide; *prd*, *paired*; Sir2, silent information regulator 2; *stg*, *string*

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rogenesis (reviewed in Fisher and Caudy 1998a; Davis and Turner 2001). Misregulation of HES family members has been linked to developmental defects and oncogenesis. In *Drosophila*, the HES family consists of Hairy and twelve other structurally related proteins, including Deadpan and seven members of the Enhancer of split complex. All members of this repressor family possess a highly conserved bHLH domain, required for DNA binding and protein dimerization; an adjacent Orange domain, which confers specificity among family members; and a C-terminal tetrapeptide motif, WRPW, which has been shown to be necessary and sufficient for the recruitment of the corepressor Groucho.

HES proteins have been shown to bind preferentially to Class C sites (CACNNG; C-box) as homodimers in vitro (Sasai et al. 1992; Tietze et al. 1992; Oellers et al. 1994; Ohsako et al. 1994; Van Doren et al. 1994). The prevailing view is that Hairy functions as a promoter-bound repressor: an intact bHLH region is required for Hairy to bind to specific DNA sites, where it then recruits cofactors to mediate its activities. Indeed, *ac* has been shown to be a direct transcriptional target of Hairy during peripheral nervous system development (Ohsako et al. 1994; Van Doren et al. 1994; Fisher et al. 1996). However, while *ftz* was identified as a genetic target of Hairy during segmentation, there is currently no evidence for Hairy binding directly to the *ftz* promoter to regulate its transcription (despite the efforts of several labs to find such an association).

A common theme among DNA-bound transcriptional regulators is the recruitment of coactivators or corepressors to carry out their functions (reviewed in Mannervik et al. 1999; Bone and Roth 2001; Urnov et al. 2001; Jepsen and Rosenfeld 2002). Three such cofactors have been identified as Hairy-interacting proteins that are required for Hairy-mediated transcriptional repression: Groucho, *Drosophila* C-terminal binding protein (dCtBP), and *Drosophila* silent information regulator 2 (dSir2) (Paroush et al. 1994; Poortinga et al. 1998; Phippen et al. 2000; Rosenberg and Parkhurst 2002). None of these cofactors bind DNA themselves, but they are brought to the DNA through their interaction with sequence-specific DNA binding repressors, such as Hairy.

Groucho was the first cofactor shown to be required for Hairy-mediated repression, where it was shown to enhance the *hairy* mutant phenotype (Paroush et al. 1994). Groucho, as well as its mammalian homologs collectively called TLEs (TLE1–4), share a similar overall domain structure (reviewed in Parkhurst 1998; Fisher and Caudy 1998b). Groucho has been proposed to utilize a chromatin remodeling mechanism through its recruitment of Rpd3 (*Drosophila* histone deacetylase 1 homolog), but the evidence for the significance of this interaction is somewhat mixed (Chen et al. 1999; Mannervik and Levine 1999; Courey and Jia 2001).

C-terminal binding protein (CtBP) family members are an interesting new class of transcriptional coregulators that encode nicotinamide adenine dinucleotide⁺-dependent (NAD⁺-dependent) acid dehydrogenases (reviewed in Turner and Crossley 2001; Chinnadurai 2002a, 2002b; Kumar et al. 2002). CtBP proteins function as context-dependent cofactors: they act as either coactivators or corepressors of transcription, with distinct regions of the CtBP protein being required for activation or repression (Nibu et al. 1998a, 1998b; Poortinga et al. 1998; Phippen et al. 2000; Chinnadurai

2002a). The mechanism of CtBP coactivation is not known. CtBP proteins, however, have also been postulated to use a chromatin-based mechanism when functioning as a corepressor for transcription: they can bind to histone deacetylases and have been shown to modify histones (Sundqvist et al. 1998; Shi et al. 2003).

Like its yeast homolog, *dSir2* encodes NAD⁺-dependent histone deacetylase activity that is required for heterochromatic silencing (Rosenberg and Parkhurst 2002; Newman et al. 2002; reviewed in Gottschling 2000; Imai et al. 2000; Denu 2003). While yeast silent information regulator 2 (Sir2) has been thought to function as a dedicated heterochromatic silencing factor, *dSir2*, and more recently the human Sir2-related protein SIRT1, have been shown to play a role in euchromatic repression by interacting with Hairy and other HES family members (Rosenberg and Parkhurst 2002; Takata and Ishikawa 2003). *dSir2* mutants are viable (Newman et al. 2002) and exhibit a dominant genetic interaction with *hairy*, resulting in derepression of *Ftz* expression (Rosenberg and Parkhurst 2002), suggesting that Sir2 in higher organisms plays a role in both euchromatic repression and heterochromatic silencing.

The choice of cofactor recruited by a particular DNA-bound repressor has been proposed to help distinguish among the mechanisms of repression employed. Despite the importance of Hairy and other HES family proteins in many developmental regulatory processes, little is known about the number and kinds of target genes they regulate. Understanding the spectrum of direct targets will be essential to addressing mechanistic questions such as how or when different cofactors are recruited. To this end, we have used the chromatin profiling technique DamID to systematically identify direct Hairy transcriptional target genes and to obtain a global view of the cofactors Hairy recruits to the various loci at which it acts.

Results

Identification of Direct Hairy Transcriptional Targets in *Drosophila* Kc Cells Using the Chromatin Profiling Technique DamID

To identify direct transcriptional targets for Hairy in vivo, we employed a powerful new chromatin profiling technique, DamID, in which *E. coli* DNA adenine methyltransferase (Dam) tethered to a chromatin binding protein leads to specific methylation of DNA adjacent to the protein binding/recruitment sites (van Steensel and Henikoff 2000; van Steensel et al. 2001). We generated a functional Dam–Hairy fusion construct under the control of the heat shock promoter to use in *Drosophila* Kc cells (see Materials and Methods). Since overexpression of Dam fusion constructs leads to a high level of nonspecific methylation (van Steensel and Henikoff 2000), only low-level leaky expression from the uninduced heat shock promoter was used: the cells were not heat shocked. Genomic DNA was isolated from the Kc cells 24 h post transfection, and methylated DNA fragments were recovered on a sucrose gradient following digestion of the genomic DNA with the methylation-sensitive enzyme DpnI. These methylated fragments were labeled with the Cy5 (Dam–Hairy fusion protein) and Cy3 (Dam alone, a control for nonspecific binding/accessibility; van Steensel and Henikoff 2000) fluorochromes, then cohybridized to a *Drosophila*

microarray chip containing approximately 6200 full-length *Drosophila* Gene Collection (DGC) cDNAs and ESTs (DGC Release 1; Rubin et al. 2000) representing roughly half of the fly cDNAs. Putative targets were identified based on the Cy5:Cy3 fluorescence ratio (van Steensel and Henikoff 2000; van Steensel et al. 2001). The DamID chromatin profiles were generated as previously described (van Steensel et al. 2001; Orian et al. 2003) and subjected to a series of statistical analyses to determine the statistically significant targets (see Materials and Methods; Datasets S1 and S2).

We identified 40 statistically significant putative direct

Hairy transcriptional targets in Kc cells (Table 1). For just over half of these putative Hairy targets, some genetic, molecular, or functional information exists, allowing us to divide them roughly into three functional categories: those affecting morphogenesis (e.g., *egghead* [*egh*], *kayak*, *pointed*, *mae*), those affecting cell cycle or cell growth (e.g., *string* (*stg*), *ImpL2*, *Idgf2*), and those with unknown/unlinked functions. Unfortunately, the two previously identified Hairy targets, *ftz* and *ac*, are not present in the DGC Release 1 cDNA set used to generate our microarray chips.

DamID was recently used to identify targets for the

Table 1. Hairy Targets Identified in Kc Cells

Gene	Name	Clone ID	Location	Function	Cofactor Recruitment			Overlap with	
					dCtBP	dSir2	Groucho	Myc	Mad
CG11676		GH15792	85F4	Unknown	–	–	–	–	–
CG31324		HL03650	97A1	Unknown	–	–	+	–	–
CG15509	<i>kayak</i>	SD04477	99B/C	bZIP transcription factor (fos homolog)	+	–	–	–	–
CG6199		LD37702	68B1	Procollagen-lysine 5-dioxygenase	+	–	–	–	–
CG10987		LD10495	19E7	Unknown	+	–	–	–	–
CG1395	<i>stg</i>	LD47579	99A5	Protein tyrosine phosphatase (cell cycle)	+	+	–	–	–
CG15009	<i>ImpL2</i>	SD07266	64A10	Ecdysone-inducible gene	+	+	–	–	–
CG4475	<i>ldgf2</i>	GH12581	36A1	Imaginal disc growth factor	+	+	–	–	–
CG15085	<i>mae</i>	LD15796	55E6	Modulator of Ets activity (ras pathway)	+	+	–	–	–
CG3705	<i>astray</i>	LD23646	67B4	Phosphoserine phosphatase	+	+	–	–	–
CG9659	<i>egh</i>	GH01085	3A6	Integral membrane component	+	+	–	–	–
CG14548	<i>E(spl)m8</i>	GH01842	96F9	bHLH transcriptional repressor (CNS, PNS)	+	+	–	–	–
CG5405	<i>KrT95D</i>	GM03043	95D	Putative Krüppel target gene	+	+	–	–	–
CG17077	<i>pointed-RC</i>	—	94E	Ets transcription factor (torso pathway)	+	+	–	–	–
CG11804	<i>ced-6</i>	LD19266	45D	PH domain	+	+	–	–	–
CG13586		SD05282	60D	Neuropeptide hormone (signaling)	+	+	–	–	–
CG6805		LD06095	53E4	Inositol triphosphate phosphatase	+	+	–	–	–
CG17278		SD04019	93B4	Serine protease inhibitor family domain	+	+	–	–	–
CG1628		LD16544	9D	Ornithine transporter	+	+	–	–	–
CG10997		LD46306	12C2	Chloride channel	+	+	–	–	–
BcDNA:LD10401		LD10401	94E6	Unknown	+	+	–	–	–
CG2765		GH12953	60E8	Unknown	+	+	–	–	–
CG3770		SD01285	60E8	Unknown	+	+	–	–	–
CG4004		LD20718	11B14	Unknown	+	+	–	–	–
CG5850		LD36053	30F4	Unknown	+	+	–	–	–
CG8954		LD22235	3400	Unknown	+	+	–	–	–
CG10433		GH10517	57F3	Unknown	+	+	–	–	–
CG13868		SD03066	56F16	Unknown	+	+	–	–	–
CG15745		LD11831	11E9	Unknown	+	+	–	–	–
—		GM02743	—	Unknown	+	+	–	–	–
—		LD16711	—	Unknown	+	+	–	–	–
CG30011	<i>gemini</i>	HL07886	46F2	Transcription factor (pointed domain)	+	+	–	–	+
CG5345	<i>Eip55E</i>	LD22255	55000	Cys/Met metabolism	+	+	–	–	+
CG15095	<i>l(2)08717</i>	GH07529	55F4	Inorganic phosphate:sodium symporter	+	+	–	–	+
CG15093		GH06781	55F2	3-Hydroxyisobutyrate dehydrogenase	+	+	–	–	+
CG12030		LD27852	61C8	UDP-glucose 4-epimerase (metabolism)	+	+	–	–	+
CG5346		LD08233	94B2	Clavimate synthetase-like	+	+	–	–	+
CG3249		GM04319	4F9	PKA anchor protein (KH domain)	+	+	–	+	+
CG30479		LD38749	51B11	Unknown	+	+	–	+	+
CG12178	<i>Nhe1</i>	LP02993	21A5	Sodium:hydrogen exchanger	+	+	–	+	–

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Drosophila Myc/Max/Mad-Mnt network of bHLH leucine zipper proteins (Orian et al. 2003), which shares many structural and functional similarities with the HES network of bHLH proteins (Gallant et al. 1996). Using the same *Drosophila* cDNA microarray chips, Orian et al. (2003) found that hundreds of binding sites are occupied by dMyc (287 targets) or dMnt (429 targets), and that their expression is modulated by dMyc in the *Drosophila* larva. Their study is consistent with a global role for Myc family proteins in modulating chromatin responsiveness of targets, and identified most of the transcriptional targets that had been found previously utilizing other approaches. As our current knowledge of direct Hairy transcriptional targets for comparison is minimal, we applied a higher stringency than Orian et al. (2003) when analyzing our Hairy DamID datasets so that we would reduce the likelihood of getting false positives. However, at this stringency we may be missing some bona fide Hairy targets. We compared the Hairy targets we identified with those identified for dMyc and dMnt using datasets analyzed at the higher statistical stringency (Figure 1). As might be expected, there was minimal overlap of Hairy targets with those identified for the transcriptional activator dMyc (three of 40 Hairy targets) (Figure 1A). There was also little overlap of Hairy targets with those identified for the transcriptional repressor dMnt (nine of 40 Hairy targets) (Figure 1B). Even when the less stringent statistics were applied to the datasets, we did not see additional overlap (data not shown). Thus, sequence-specific DNA binding factors are exhibiting binding specificity in the DamID assay, and the 40 statistically significant putative direct Hairy transcriptional targets we identified are what might be expected for a nonglobally acting sequence-specific DNA binding developmental repressor.

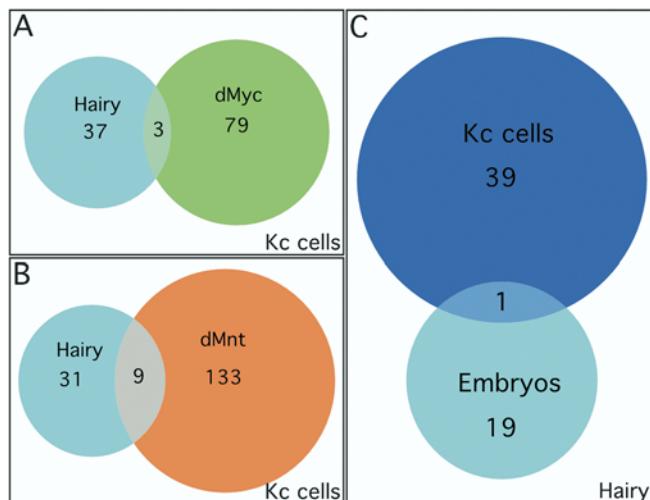


Figure 1. Hairy Binds to a Specific Set of Transcriptional Targets

(A and B) Comparison of DamID-identified targets for Hairy with the *Drosophila* Myc and Mad/Mnt family proteins. Venn diagram comparing DamID-identified Hairy downstream targets in Kc cells compared to the transcriptional activator dMyc (A) and the transcriptional repressor dMnt (B).

(C) Venn diagram comparing DamID-identified Hairy targets from Kc cells and embryos.

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Identification of Direct Hairy Transcriptional Targets in Early Embryos Using DamID

Since Hairy is part of the segmentation gene transcriptional regulatory cascade, we expected to find segmentation-related transcription factors as downstream targets of Hairy. The putative Hairy targets we identified in Kc cells do not fulfill this expectation, but rather suggest roles for Hairy in cell cycle, cell growth, and morphogenesis; these putative targets are likely targets for Hairy during its other developmental roles. This could be because of cellular context (i.e., Kc cells are thought to be embryonic neuronal stem cell in origin and may reflect Hairy's later role in neurogenesis rather than segmentation), or because only half of the *Drosophila* cDNAs are present on the chip (and the ones responding to Hairy during segmentation are not in this subset), or because the mechanism by which Hairy acts during segmentation is different than expected. To begin distinguishing among these possibilities, we used the DamID approach to identify Hairy targets in *Drosophila* embryos during segmentation.

Towards this aim, we generated functional transgenic flies carrying a UAS-Dam or UAS-Dam-Hairy fusion gene construct (see Materials and Methods). As with the Kc cells, we did not drive overexpression of these Dam fusion constructs, but rather relied on the leaky expression from the minimal promoter of the pUASp vector. Genomic DNA was harvested from 2–6-h embryos (at and just after peak Hairy expression during segmentation), then used to generate probes for the microarray chips, similar to the procedure used for the Kc cells (see Materials and Methods; Datasets S1 and S3).

We identified 20 putative direct Hairy targets from the 2–6-h embryos, which fell into four broad functional categories: transcription factors, cell cycle or cell growth, morphogenesis, and unknown/unlinked functions (Table 2). When compared to the 40 Hairy targets identified in Kc cells, we found that only one target, *egh*, overlapped between the datasets (Figure 1C). This result suggests, perhaps not surprisingly, that transcriptional targets exhibit context dependence/tissue specificity, and that the DamID approach is sensitive to developmental context/tissue specificity.

Taken together, the DamID profiles for Hairy targets from Kc cells and embryos identified 59 potential new direct targets of Hairy regulation. Importantly, one of the putative Hairy targets in embryos, *paired* (*prd*), is a homeobox-encoding transcription factor known to function in segmentation (cf. Baumgartner and Noll 1990).

The Expression of Potential Target Genes Depends on Hairy Regulation In Vivo

Direct Hairy targets would be expected to exhibit altered expression in a *hairy* mutant background compared to wild-type. For a subset of targets from both the Kc cell and embryo DamID experiments, we performed whole mount RNA in situ hybridization on wild-type and *hairy*^{7H} embryos (Figure 2 and data not shown). For embryo targets, we examined early embryos representing the same stages used for the DamID analysis. In keeping with our primary focus on Hairy's role in segmentation, we chose as the subset of Kc target genes to examine genes known to be expressed in the embryo (but not necessarily as early as the embryo targets), since we would not expect all of the Kc cell targets to be expressed during embryogenesis. In all cases examined, the

Table 2. Hairy Targets Identified in Embryos (2–6 h)

Gene	Name	Clone ID	Location	Function
CG9659	<i>egh</i>	GH01085	3A6	Integral membrane component
CG6716	<i>prd</i>	GH22686	33C3	Homeodomain protein (pair-rule gene)
CG8643	<i>regular</i>	GH06282	44D4	Transcription factor
CG17678	<i>concertina</i>	—	40F	G-alpha-like protein
CG1772	<i>dacapo</i>	LP07247	46B1	Cyclin-dependent protein kinase inhibitor
CG9900	<i>mitotic15</i>	SD07771	3A	Mitotic chromosome segregation
CG4123	<i>Mipp1</i>	GM09242	73A	Inositol/phosphatidylinositol phosphatase
CG9704	<i>Neurotactin</i>	LD22004	73C4	Esterase/lipase/thioesterase
CG10241	<i>Cyp6a17</i>	GH10635	51D1	Cytochrome P450
CG8636		LD24347	3A7	Translation initiation factor (zinc finger)
CG11907		LD19162	21C	Nucleoside transporter
CG10764		LP05421	54B16	Serine proteases
CG2233		GH20802	7D2	Unknown
CG32605		GM04658	12D	Unknown
CG32331		LD40851	62A2	Unknown
—		CK00534	—	Unknown
—		CK01134	—	Unknown
—		LD43581	—	Unknown
—		CK01099	—	Unknown
—	28SPP1			

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alterations in the levels, as well as spatial and temporal patterns, of putative target gene expression were consistent with derepression in a *hairy* mutant background (Figure 2). For example, as previously described (Lehman et al. 1999), segmental expression of *stg* is altered (expanded) in a *hairy* mutant background (Figure 2C and 2D). Similarly, for *prd*, there is a failure of stripe sharpening consistent with a role for Hairy in *prd* repression and stripe maintenance (Figure 2A and 2B; Gutjahr et al. 1993).

hairy Exhibits Dominant Genetic Interactions with Mutants Encoding Target Genes and Affects *stg-lacZ* Reporter Expression

If Hairy is a direct regulator of a particular target gene, genetic interaction might be expected between *hairy* and a mutant corresponding to this putative target. Reduction of *hairy* dose might be expected to deregulate the expression of its target gene, resulting in increased or spatially aberrant expression of its target gene. We examined seven of the 15 Hairy targets for which mutant alleles are available for genetic interaction with *hairy* (Table 3). In all seven cases, we observed dominant genetic interactions where a reduced number of transheterozygous progeny survive (i.e., synthetic lethality). Embryos from mothers heterozygous for either *hairy* (*hairy*⁺) or its target gene (i.e., *prd*⁺) alone were viable. The reduction of Hairy in this target-gene-sensitized background allows inappropriate target gene regulation (i.e., target gene expression in spatial domains where it should not be expressed, with subsequent embryo lethality).

For one Hairy target identified in Kc cells, *stg*, a series of transgenic lines have been generated in which *lacZ* expression is driven from different promoter fragments (Lehman et al.

1999). For *stg* to be a direct transcriptional target of Hairy, we would expect Hairy to bind to the *stg* promoter. To narrow down regions of the *stg* promoter sensitive to Hairy, we examined the expression of four of these *stg-lacZ* reporter genes in *hairy* mutant and wild-type backgrounds. Sequence analysis of the promoter fragments for each of the four reporter genes revealed the presence of canonical Hairy binding sites in two of them (p_{stg} β-E4.9 and p_{stg} β-E6.4), but not the other two (p_{stg} β-E2.2 and p_{stg} β-E6.7). Consistent with the presence of Hairy binding sites, the *lacZ* expression from p_{stg} β-E4.9 and p_{stg} β-E6.4, but not from p_{stg} β-E2.2 or p_{stg} β-E6.7, was derepressed (expanded) in a *hairy* mutant background compared to wild-type (Figure 3; data not shown). We mutated the C-box (Hairy binding site) in the p_{stg} β-E4.9 reporter construct (CACGCG→CTCGCA) to generate p_{stg} β-E4.9^{Ahairy}. This mutation abolishes Hairy binding in vitro (see next section and Materials and Methods). Wild-type flies carrying this p_{stg} β-E4.9^{Ahairy} reporter exhibit the same *lacZ* derepression as observed for the original p_{stg} β-E4.9 reporter when in a *hairy* mutant background, indicating that the derepression is due to Hairy binding (Figure 3G).

Hairy Binds Directly to Target Genes

Hairy has been shown to bind at Class C sequences (ggCACGCG^A_{LC}) that contain the canonical core Hairy site (CACGCG). We searched for this consensus site within the promoter and transcribed regions of three Hairy targets: *stg*, *egh*, and *prd*. We identified one site in *prd*, three in *egh*, and four in the *stg* genomic region (Figure 4A). In the latter case, we focused on the site within the 4.9-kb promoter fragment, as its segmental expression was derepressed in a *hairy* mutant background (see above). We tested whether the identified

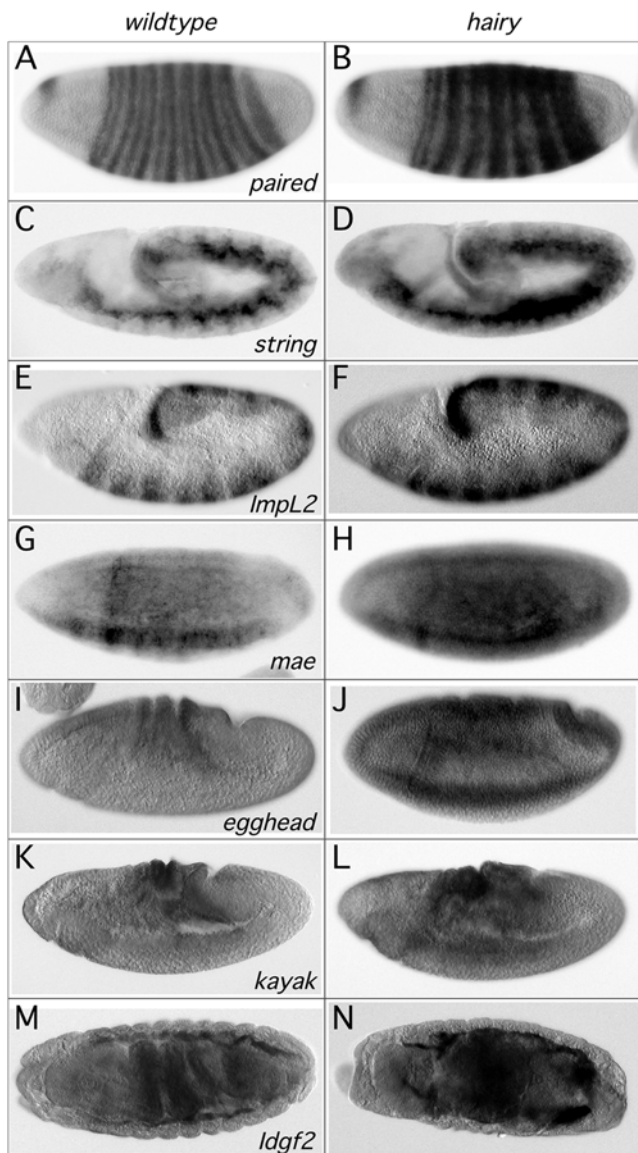


Figure 2. Expression of Hairy Target Genes Is Disrupted in *hairy* Mutant Embryos

Whole mount in situ hybridization on wild-type (A, C, E, G, I, K, and M) or *hairy*^{7H} mutant (B, D, F, H, J, L, and N) embryos with probes recognizing *prd* (A and B), *stg* (C and D), *ImpL2* (E and F), *mae* (G and H), *egh* (I and J), *kayak* (K and L), or *ldgf2* (M and N). Anterior is to the left. Dorsal is up, except in (M) and (N), which are dorsal views. DOI: 10.1371/journal.pbio.0020178.g002

sites are direct Hairy binding sites in electromobility shift assays (EMSAs), utilizing bacterially purified full-length Hairy protein and ³²P-labeled oligos containing the appropriate Hairy binding sites (see Materials and Methods). The C-box within the *ac* promoter, a bona fide Hairy target (Ohsako et al. 1994; Van Doren et al. 1994), served as our positive control. A slow migrating complex was observed when the *ac* probe was incubated with GST-Hairy protein, but not with GST alone (Figure 4B, compare lanes 2 and 3). This binding is specific: the complex is competed by excess unlabeled wild-type *ac* oligo, but not by excess mutated *ac* oligo (Figure 4B, lanes 4 and 5, respectively). Similar assays showed direct and specific binding to the sole C-box site within the *prd* promoter, as well

as to the site within the *stg* 4.9-promoter region (Figure 4C). While an oligo containing the wild-type Hairy binding site efficiently competes with Hairy binding to the *stg* 4.9-promoter region in EMSAs, an oligo encoding the mutated Hairy site used in the *pstg* β-E4.9^{Δhairy} reporter is unable to compete (Figure 4D). Three putative sites were identified within the *egh* promoter. Hairy binding to these sites was differential, and can be summarized as *egh1* > *egh3* > *egh2* (Figure 4E; compare lanes 3, 7, and 11). This preferential binding may reflect sequences flanking the core C-box (CACGCG; see Figure 4A). Indeed, experiments with the related fly Enhancer of split proteins have shown that even subtle sequence changes within the core C-box or flanking sequences have dramatic consequences for the overall range of proteins that can bind in vivo (Jennings et al. 1999). We have used several bioinformatics approaches to analyze Hairy target gene promoters, to determine if there are conserved sequences flanking the core Hairy binding sites or association of the Hairy binding sites with other transcription factor binding sites as defined by the TRANSFAC database that correlate with the context dependence of Hairy binding. However, we have been unable to uncover any common features of regulation, perhaps because of the relatively small sample size of Hairy targets for these types of approaches (see Materials and Methods; data not shown).

Hairy Binds to Specific Sites on Polytene Chromosomes

To confirm the genomic loci associated with Hairy in vivo, we examined binding of endogenous Hairy to third instar larval salivary gland polytene chromosomes using antibodies to Hairy (Figure 5). We identified approximately 120 strongly staining sites for Hairy (Figure 5). This is likely an underestimate as some bands stain more intensely than others and likely represent more than one closely spaced binding site. Hairy binding sites are, for the most part, distributed evenly along all chromosome arms (Figures 5 and 9A).

Since there are a relatively small number of Hairy binding sites on the polytene chromosomes, the location of the bands can be determined cytologically with relatively high resolution. While we have not been able to unambiguously assign all of the approximately 120 binding sites cytologically, we examined whether Hairy staining corresponds to the targets identified in the Kc cells and embryo DamID experiments. There are 39 out of 40 Kc cell and 20 out of 20 embryo targets that map cytologically to regions that correspond to Hairy binding sites (e.g., Figure 6A–6F). Thus, while tissue or developmental specificity appear to be lost, polytene chromosomes provide a reliable indicator for Hairy DNA binding targets. Note the presence of Hairy binding at the tip of the X chromosome, the cytological location of the direct Hairy transcriptional target *ac* (Figure 6A). Interestingly, we were unable to detect Hairy binding at position 84A, the cytological location for *ftz* (Figure 6B). Hairy binding was also detected at the cytological location for *stg* (Figure 6C) and *egh* (Figure 6D), as well as at 33C, the cytological location of *prd* (Figure 6E). Recent work established a role for Hairy in regulating salivary gland tube morphology that genetically depends, in part, on repression of *huckebein* (*hkb*), a zinc-finger-encoding transcription factor (Myat and Andrew 2002). It is not yet known if Hairy's repression of *hkb* is direct or not. *hkb* is not in the DGC Release1 cDNA set used to generate our microarray chips, but we do find that one of the

Table 3. Dominant Genetic Interactions between *hairy* and Mutants Corresponding to Its Putative Downstream Targets

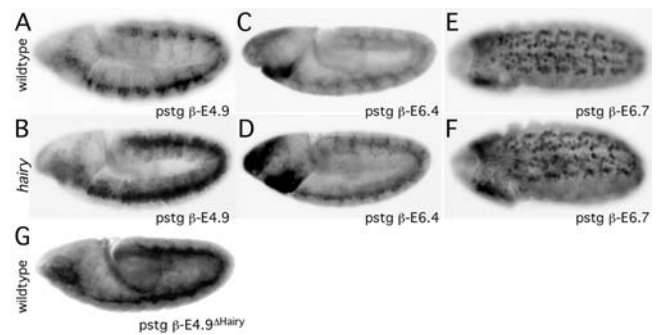
Female	Male	Percent Embryonic Lethality	Number Scored
+/+	h ^{7H} /+	1	433
+/+	h ⁱ²² /+	2	483
prd ^{2.45.17} /+	+/+	2	531
prd ^{2.45.17} /+	h ^{7H} /+	16	676
prd ^{2.45.17} /+	h ⁱ²² /+	14	439
pnt ^{Δ88} /+	+/+	0	375
pnt ^{Δ88} /+	h ^{7H} /+	24	466
pnt ^{Δ88} /+	h ⁱ²² /+	12	708
mae ^{K06602} /+	+/+	2	383
mae ^{K06602} /+	h ^{7H} /+	24	573
mae ^{K06602} /+	h ⁱ²² /+	18	726
egh ⁷ /FM7a ^a	+/+	3	471
egh ⁷ /FM7a	h ^{7H} /+	13	475
egh ⁷ /FM7a	h ⁱ²² /+	10	630
stg ^{AR2} /+	+/+	3	484
stg ^{AR2} /+	h ^{7H} /+	13	400
stg ^{AR2} /+	h ⁱ²² /+	17	667
ImpL2/ImpL2	+/+	6	347
ImpL2/ImpL2	h ^{7H} /+	26	478
ImpL2/ImpL2	h ⁱ²² /+	31	455
rgr ^{KG03110} /+	+/+	2	422
rgr ^{KG03110} /+	h ^{7H} /+	22	822
rgr ^{KG03110} /+	h ⁱ²² /+	12	745

^aHomozygous *egh*⁷ exhibits pupal (not embryonic) lethality.
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strong Hairy binding sites maps to 82A on polytene chromosomes, the cytological location of *hkb* (see Figure 6F). Consistent with our identification of *stg* as a Hairy target, derepression of C-box-containing *stg-lacZ* reporter lines, and gel shift assays, we detect a new band of Hairy staining in chromosomes from larvae carrying the *stg-lacZ* (pstg β-E4.9) reporter at cytological location 1F, the transgene insertion site (Figure 6G–6J; see Materials and Methods).

Identification of Targets for Recruitment of the Transcriptional Cofactors Groucho, dCtBP, and dSir2

As with other sequence-specific DNA binding transcription factors, Hairy recruits cofactors to carry out its functions. One of the major questions in the field concerns how and when particular cofactors are recruited. It has been technically challenging to address this question with current methods such as ChIP assays, since cofactor association may be transient, unstable, or far removed from the DNA binding protein. Utilizing expression-based microarray analysis is also not easy, because of the difficulty in sorting direct from indirect interactions with such widely recruited cofactors. To circumvent these technical issues and as a first step towards understanding the rules governing Hairy cofactor recruitment, we used the DamID approach to determine if the three known Hairy cofactors, Groucho, dCtBP, and dSir2, are recruited to all or a subset of Hairy targets. We generated

**Figure 3.** *hairy* Affects *stg-lacZ* Reporter Expression

(A–F) β-galactosidase expression from the *stg-lacZ* reporter lines pstg β-E4.9 (A and B), pstg β-E6.4 (C and D), and pstg β-E6.7 (E and F) in wild-type (A, C, and E) and *hairy* mutant (B, D, and F) embryos. Note the expanded (de-repressed) lacZ expression in the *hairy* mutant background compared to wild-type for the E4.9 and E6.4 lines (compare [B] to [A] and [D] to [C], respectively).

(G) β-galactosidase expression from the *stg-lacZ* reporter line pstg β-E4.9^{ΔHairy} (same as the reporter construct shown in [A], but with a Hairy binding site mutation) in a wild-type background. Note the expanded (de-repressed) lacZ expression (compare with [A]). Anterior is to the left. Dorsal is up in (A–D) and (G), whereas the ventral surface is shown in (E–F).
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Dam fusions to Groucho and dCtBP (see Materials and Methods). The Dam–dSir2 fusion construct was described previously (van Steensel et al. 2001). While none of these cofactors binds DNA on its own, they are recruited to the DNA through their interaction with sequence-specific DNA binding proteins such as Hairy. Using the same procedure and statistical analyses used for the identification of Hairy targets in Kc cells (see Material and Methods; Datasets S1 and S4–S6), we identified 155 loci that recruit Groucho, 496 loci that recruit dCtBP, and 107 loci that recruit dSir2 in Kc cells (Figure 7; Datasets S7–S9). Comparison for overlap between these cofactor datasets and that of Hairy from Kc cells showed that, surprisingly, only one of the putative Hairy targets we identified overlaps with Groucho recruitment (Figure 7A and 7D). The majority of Hairy targets, however, overlap with dCtBP (38/40; Figure 7B and 7D), and most of these also overlap with dSir2 (34/40; Figure 7C and 7D). At present, we cannot rule out the possibility that a protein unrelated to Hairy is recruiting these cofactors to a given putative Hairy target. Interestingly, dCtBP and dSir2 appear to colocalize at loci outside the subset of putative Hairy targets (90% of dSir2 targets overlap with those of dCtBP; Figure 7D).

Hairy Target Gene Expression Depends on Hairy Cofactor Regulation In Vivo

If particular Hairy targets require specific cofactors to be appropriately regulated, we would expect their expression to be altered (deregulated) in a cofactor mutant background. We performed RNA in situ hybridization for two Hairy Kc cell targets that differentially recruit Groucho, dCtBP, and dSir2. We chose to examine the expression of two *hairy* targets that are expressed relatively early in the embryo since these cofactors are used in a number of developmental systems and exhibit severe morphological phenotypes when their activity is removed maternally (cf. Phippen et al. 2000). Consistent with a requirement for dCtBP and dSir2, *stg* expression is

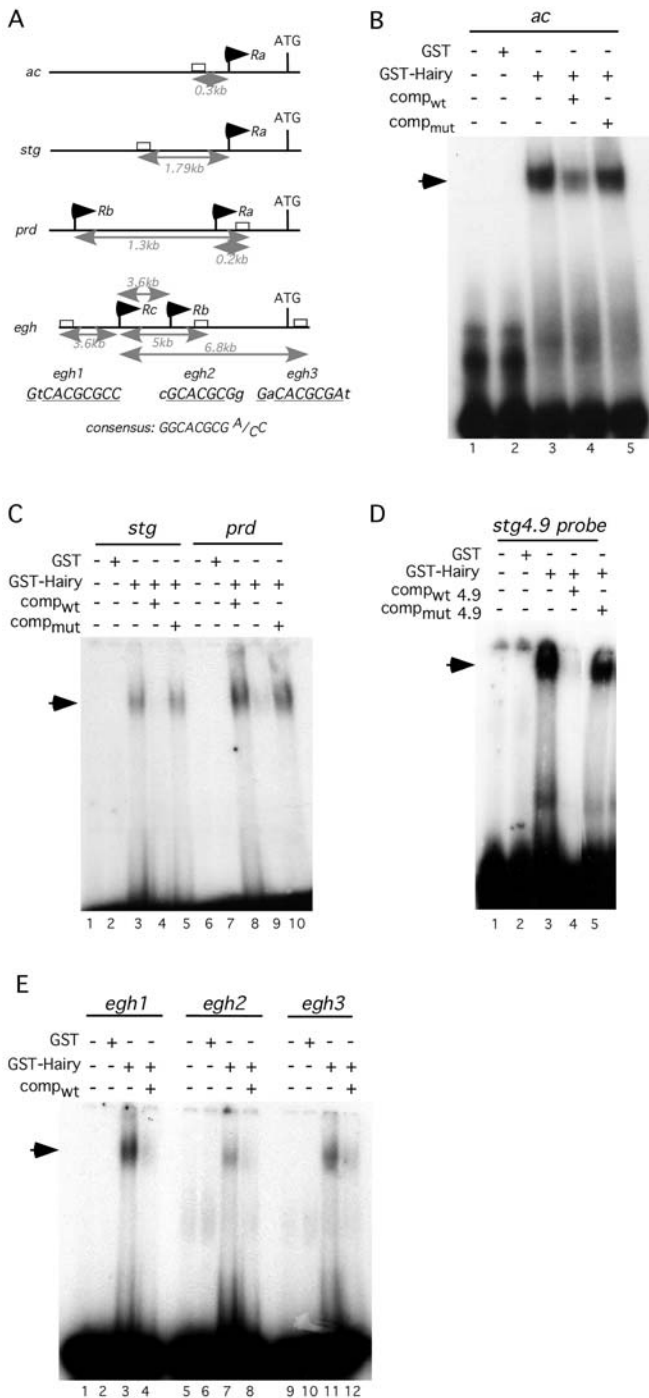


Figure 4. Binding of Hairy to Class C (C-Box) Sites in Putative Targets In Vitro

(A) Schematic diagram (not to scale) of C-boxes within putative Hairy targets. C-boxes (Hairy binding sites) are denoted by white boxes, black arrows indicate transcription start sites (Ra, Rb, and Rc), ATG denotes the initiating methionine, and capital letters indicate bases matching with the Hairy consensus C-box. The distances in kilobases of the C-boxes from transcription start sites are noted in gray.

(B) EMSA with either GST or GST-Hairy and the *ac* h/E-1 oligonucleotide. Lane 1, probe alone; lane 2, binding to probe by GST; lanes 3–5, binding to probe by GST-Hairy. In lanes 4 and 5, binding to probe by GST-Hairy was in the presence of competitor unlabeled oligos. An arrow indicates the Hairy-DNA complex; comp_{wt} and comp_{mut} indicate wild-type and mutated cold probes, respectively.

(C) EMSA with either GST or GST-Hairy to the C-boxes within the *stg*

and *prd* genes. Lanes 1–5, GST and GST-Hairy binding to the *stg* C-box (location: 25072658); lanes 6–10, GST and GST-Hairy binding to the *prd* C-box. (location: 12074032). Lane order and annotations are as in Figure 4B.

(D) EMSA with GST-Hairy to the same C-box within the *stg* 4.9-kb genomic fragment is not competed by the presence of mutant competitor unlabeled oligo. Lane 1, probe alone; lane 2, binding to GST; lane 3, binding to probe by GST-Hairy; lanes 4 and 5, binding to probe by GST-Hairy in the presence of wild-type and mutant competitor unlabeled oligos, respectively.

(E) Differential binding to C-boxes within the *egh* gene. EMSA with either GST or GST-Hairy to C-boxes within the *egh* promoter and transcribed region. Binding to three putative C-box sites is shown: *egh1* (location: 2341609), *egh2* (location: 2350367), and *egh3* (location: 2352168). Lanes 1, 5, and 9: probe alone; lanes 2, 6, and 10: binding to probes by GST; lanes 3, 7, and 11: binding to probes with GST-Hairy. Lanes 4, 8, and 12: binding with GST-Hairy in the presence of unlabeled wild-type competitor. C-box locations and promoter information generated using Apollo (Berkeley *Drosophila* Genome Project).

DOI: 10.1371/journal.pbio.0020178.g004

derepressed in *dCtBP* and *dSir2*, but not *groucho* mutant backgrounds (Figure 8A–8D). Similarly, consistent with a requirement for dCtBP alone, *kayak* expression is expanded in *dCtBP*, but not in *groucho* or *dSir2* mutant backgrounds (Figure 8E–8H). While we cannot extrapolate the cofactor recruitment requirements from Kc cells to embryos, we used in situ hybridization as a prediction for cofactor recruitment for the embryo target, *prd*. We examined the expression of *prd* in cofactor mutant backgrounds and found that *prd* expression is altered in *groucho* and *dCtBP*, but not *dSir2*, mutant backgrounds (Figure 8I–8L), suggesting that *prd* may represent a minority of Hairy targets that could recruit both Groucho and dCtBP. Consistent with this finding, we find both Groucho and dCtBP staining on polytene chromosomes at the cytological location for *prd* (data not shown).

The Transcriptional Cofactors Groucho, dCtBP, and dSir2 Are Recruited to Specific Sites on Polytene Chromosomes

When DamID data for the three Hairy cofactors and Hairy itself are graphically projected onto chromosomes, several interesting features come to light (Figure 9A). For example, while Groucho and dCtBP are distributed along all the chromosomes, dSir2 shows region- and chromosome-specific binding (e.g., there are more dSir2 sites on Chromosome 2R than on Chromosome 3L). To confirm loci associated with recruitment of the different cofactors in vivo, we examined the localization of endogenous Groucho, dCtBP, and dSir2 on wild-type third instar larval salivary gland polytene chromosomes using antibodies to Groucho, dCtBP, and dSir2, respectively (Figure 9B–9D). Consistent with the relative numbers of targets identified for each of the cofactors by the DamID approach, we find many more sites for dCtBP than either Groucho or dSir2. Also consistent with our DamID findings, Groucho overlaps with Hairy at only a small number of the Hairy binding sites (Figure 9E), whereas dCtBP overlaps with the majority of Hairy binding sites (Figure 9F). Differences in distribution for the cofactors observed by DamID are reflected on the polytene staining patterns. For example, our DamID data suggest that the distal portion of Chromosome 2L has more sites for dCtBP than the proximal half of the chromosome. This observation is reflected in dCtBP recruitment on the polytene chromosomes as well (Figure 9F). Likewise, as predicted from the DamID data, dSir2 staining on the polytene chromosomes exhibits region-

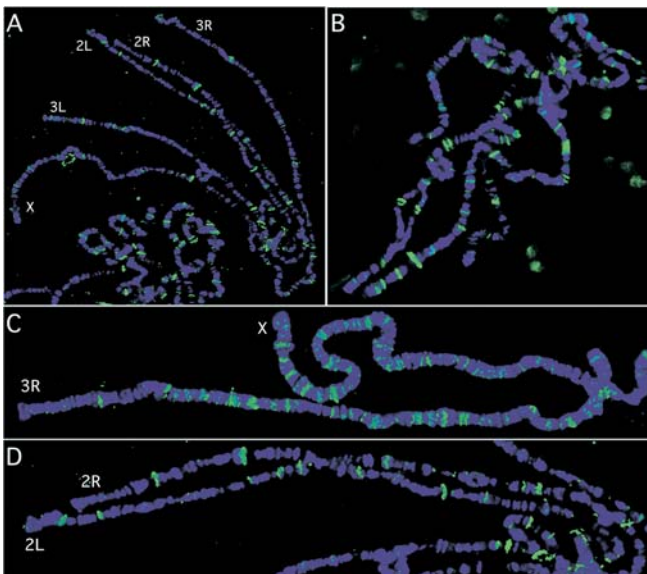


Figure 5. Hairy Binds to Specific Loci on Polytene Chromosomes
 (A and B) Hairy staining (green) on third instar larval salivary gland polytene chromosome sets counterstained with DAPI (blue) to visualize the chromosomes.
 (C and D) Higher magnification of chromosome arms X, 3R (C) and 2L, 2R (D).
 DOI: 10.1371/journal.pbio.0020178.g005

specific association in which some chromosomes and chromosomal regions exhibit a high degree of staining, while other whole chromosomes exhibit very little staining (Figure 9D and 9G–9I; Rosenberg and Parkhurst 2002).

Discussion

We have known for almost two decades that Hairy plays a pivotal role in the segmentation hierarchy, as well as other developmental processes, but the details of Hairy action have not been easy to tease apart. An important step in understanding the molecular mechanisms surrounding Hairy-mediated repression was made with the identification of Groucho as a Hairy binding protein (Paroush et al. 1994). One of the key remaining questions regarding the mechanism(s) of repression employed by Hairy concerns the identities of its direct transcriptional targets. We have employed a novel chromatin profiling approach, DamID, to effectively identify a total of 59 potentially direct Hairy targets from 2–6-h embryos and Kc cells. As expected of direct targets, these genes show Hairy-dependent expression, are detected as Hairy binding sites in vivo on polytene chromosomes, and have consensus C-box-containing sequences that are directly bound by Hairy in vitro. While the DamID approach had previously been used only in Kc cells, we found that this technique is also powerful when utilizing transgenic embryos that carry fusions of the protein of interest to the Dam methylase. As target genes are likely context dependent, the use of embryos makes it possible to choose the precise time or place of development to be examined, as well as allowing the analysis to take place in an organismal context.

The 59 putative Hairy targets we identified in the embryo and Kc cell DamID experiments correspond to bands of Hairy immunostaining on polytene chromosomes, suggesting

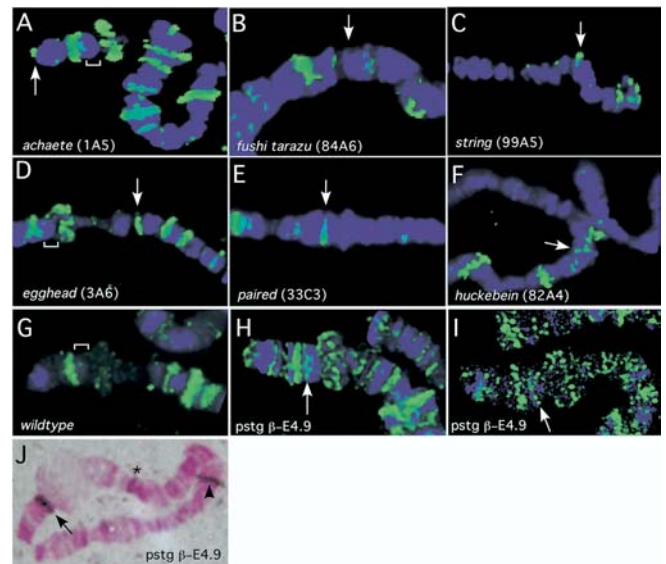


Figure 6. Hairy Binds to Putative Target Loci on Polytene Chromosomes
 (A) Hairy binds to polytene region 1A, the location of the Hairy target, *ac*.
 (B) Hairy is not found at 84A, the cytological location for *ftz*.
 (C–F) Hairy also binds to polytene region 99A, the location of *stg* (C); polytene region 3A, the location of *egh* (D); polytene region 33C, the location of *prd* (E); and polytene region 82A, the location of *hbb* (F).
 (G–I) Hairy is recruited to the insertion site for the *pstg* β -E4.9 reporter construct (arrow in [H] and [I]). Compare to the equivalent region of wild-type X chromosomes marked by brackets in (A), (D), and (G).
 (J) In situ hybridization to polytene chromosomes from *pstg* β -E4.9 larvae showing that this line has two insertions on the X chromosome at 1F and 6C. The probe also recognizes sequences to the endogenous *white* locus (asterisk).
 DOI: 10.1371/journal.pbio.0020178.g006

that the polytene chromosome staining faithfully represents Hairy binding. Polytene chromosomes are functionally similar in transcriptional activity and display factor/cofactor binding properties similar to chromatin of diploid interphase cells, despite their DNA endoreplication (Hill et al. 1987; Andrew and Scott 1994; Hill and Mott 2000; Pile and Wassarman 2000, 2002). Since the microarray chips we used contain roughly half of *Drosophila* cDNAs, we estimate the actual number of Hairy targets to be approximately twice that number (i.e., 118 targets). This predicted number of Hairy targets is close to the approximately 120 strongly staining sites we observe on polytene chromosomes. Of the 59 putative Hairy targets we identified in both the Kc cell and embryo DamID experiments, 58 correspond to bands of Hairy staining on the polytene chromosomes, suggesting that polytene chromosome staining is representing Hairy binding sites without regard to tissue specificity. It is not yet clear what is limiting Hairy accessibility in different tissues or why Hairy's access does not appear to be limited in salivary glands. It may be that polytene chromosome organization necessitates a looser chromatin structure or that the large number of factors that seem to be endogenously expressed in salivary glands affects accessibility. Ultimately, additional confirmation of the DamID and polytene staining correspondence will require microarray tiling chips containing overlapping genomic DNA fragments; however, such genomic DNA tiling chips are currently unavailable.

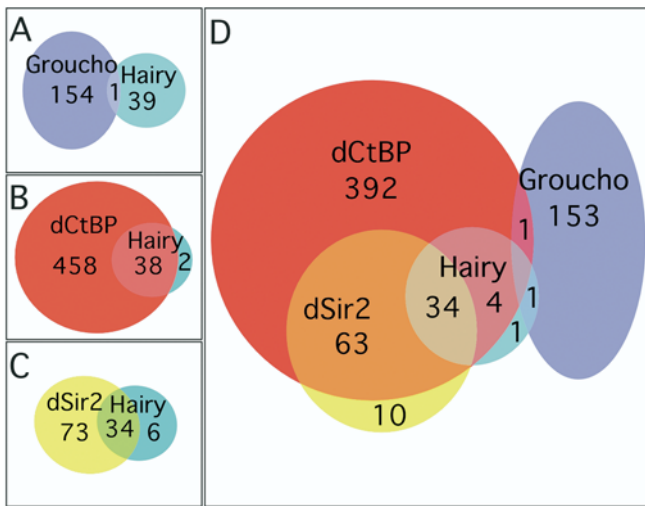


Figure 7. Hairy Overlaps with Cofactors Differentially
(A–C) Venn diagram showing the overlap between Hairy targets and those loci also binding to the cofactors Groucho (A), dCtBP (B), and dSir2 (C).
(D) Venn diagram showing combined overlaps of Hairy with its three known cofactors.
DOI: 10.1371/journal.pbio.0020178.g007

Van Steensel and Henikoff (2000) showed that DNA methylation by tethered Dam spreads up to a few kilobases from the point where it is brought to the DNA. We were concerned in the beginning that we might miss Hairy targets if the DNA fragments of 2.5 kb or less that we recovered for probes were far away from the start of the transcribed region, especially since the *Drosophila* microarray chip we used was generated using full-length cDNAs. Indeed, as Hairy has been described as a long-range repressor (Barolo and Levine 1997), it is likely to bind at a distance from the transcription start site. However, the targets we identified by DamID in both Kc cells and in embryos correspond closely to the Hairy staining pattern on polytene chromosomes. As is the case for Hairy, the distribution of DamID-identified loci that recruit the long-range repression-mediating Groucho corepressor (Zhang and Levine 1999) corresponds well with the distribution of Groucho binding sites on polytene chromosomes. Our results suggest that there is a higher-order structure to the promoter that is allowing factors that bind far upstream of the transcription start site to have physical access to the transcribed region (i.e., DNA looping; reviewed in Ogata et al.

2003) or that Hairy does not bind as far away from the transcription start site as it has been proposed to do.

Hairy Targets

Hairy is needed at multiple times during development, where it has primarily been associated with the regulation of cell fate decisions. During embryonic segmentation, *ftz* has long been thought to be a direct Hairy target. However, the order of appearance of *ftz* stripes is not inversely correlated with those of Hairy, as would be expected if *ftz* stripes are generated by Hairy repression (Yu and Pick 1995). While we were unable to assess *ftz* as a direct Hairy target using DamID, we did not find evidence for *ftz* being a direct Hairy target based on the association of Hairy with polytene chromosomes. Indeed, the evidence suggesting that *ftz* is a direct target of Hairy is based on timing, i.e., that there is not enough time for another factor to be involved (cf. Ish-Horowicz and Pinchin 1987). As the half-life of the pair-rule gene products is very short (less than 5 min; Edgar et al. 1986), it is possible that additional factors could be acting and that the interaction between Hairy and *ftz* is indirect.

Interestingly, one of the Hairy targets we identified in embryos is the homeobox-containing transcriptional regulator, *prd*. Pair-rule genes have been split into two groups: primary pair-rule genes mediate the transition from non-periodic to reiterated patterns via positional cues received directly from the gap genes, whereas secondary pair-rule genes take their patterning cues from the primary pair-rule genes and in turn regulate the segment polarity and homeotic gene expression. The transcriptional regulator *prd* was originally categorized as a secondary pair-rule gene since its expression is affected by mutations in all other known pair-rule genes. However, *prd* stripes were subsequently shown to require gap gene products for their establishment, and the *prd* locus has the modular promoter structure associated with primary pair-rule genes (Baumgartner and Noll 1990; Gutjahr et al. 1993). Thus, *prd* has properties of both primary and secondary pair-rule genes and is a good candidate to directly mediate Hairy's effects on segmentation. We found that Hairy can specifically bind to C-box sequences in the *prd* promoter and interacts genetically with *prd*. Further experiments will be required to determine if Paired in turn binds to the *ftz* promoter, such that the order of regulation would be Hairy > *prd* > *ftz*.

In addition to identifying potential targets for Hairy in segmentation, we identified targets that implicate Hairy in other processes including cell cycle, cell growth, and

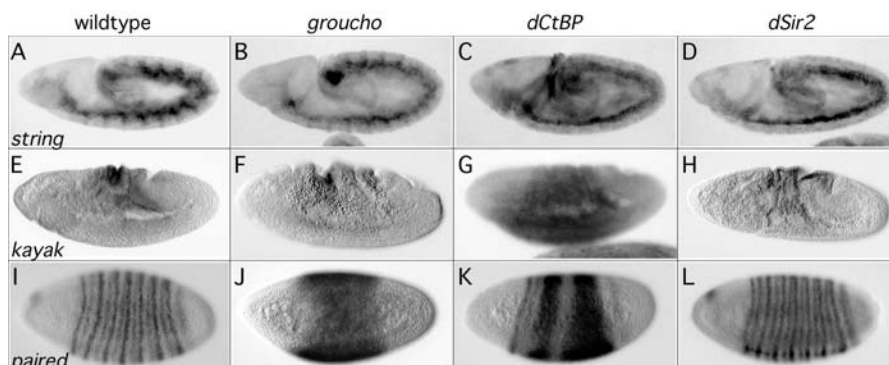


Figure 8. Hairy Target Gene Expression Is Disrupted in the Mutant Background of the Cofactors Associated with a Particular Target

Whole mount in situ hybridization on wild-type (A, E, and I), *groucho* germline clone (B, F, and J), *dCtBP* germline clone (C, G, and K), and *dSir2* mutant (D, H, and L) embryos with probes recognizing *stg* (A–D), *kayak* (E–H), or *prd* (I–L). Anterior is to the left. Dorsal is up.
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morphogenesis. The group of targets implicating Hairy in the regulation of morphogenesis includes: *concertina*, a G-alpha protein involved in regulating cell shape changes during gastrulation (Parks and Wieschaus 1991); *kayak*, the *Drosophila* Fos homolog involved in morphogenetic processes such as follicle cell migration, dorsal closure, and wound healing (Riesgo-Escovar and Hafen 1997; Dequier et al. 2001; Dobens et al. 2001; Ramet et al. 2002); *pointed* and *mae*, both of which function in the *ras* signaling pathway to control aspects of epithelial morphogenesis (cf. Beitel and Krasnow 2000; Baker et al. 2001; James et al. 2002); *egh*, a novel, putative secreted or transmembrane protein proposed to play a role in epithelial morphogenesis (Goode et al. 1996); and *Mipp1*, a phosphatase required for proper tracheal development (Ebner et al. 2002).

Hairy has been thought to be involved mostly in the regulation of cell fate decisions. However, mosaic experiments in the eye imaginal disc have suggested that Hairy may also play a role in the regulation of cell cycle or cell growth (Brown et al. 1995). Consistent with this, another group of Hairy targets implicates Hairy in the regulation of cell cycle or cell growth; this group includes *stg*, the *Drosophila* Cdc25 homolog (cf. Lehman et al. 1999); *dacapo*, a cyclin-dependent kinase inhibitor related to mammalian p27^{kip1}/p21^{waf1} (Lane et al. 1996; Meyer et al. 2002); *IDGF2*, a member of a newly identified family of growth-promoting glycoproteins (Kawamura et al. 1999); and *ImpL2*, a steroid-responsive gene of the secreted immunoglobulin superfamily that functions as a negative regulator of insulin signaling (Garbe et al. 1993; Andersen et al. 2000; Montagne et al. 2001; Tapon et al. 2001; Johnston and Gallant 2002). Consistent with a role for Hairy in growth signaling, mammalian HES family proteins have been linked to insulin signaling (Yamada et al. 2003).

Since cells that are dividing or proliferating cannot simultaneously undergo the cell shape changes and cell migrations required for morphogenetic movements, Hairy may be required to transiently pause the cell cycle in a spatially and temporally defined manner, thereby allowing the cell fate decisions regulated by the transcription cascade to be completed. As Hairy is itself spatially and temporally expressed, Hairy must be only one of several genes necessary to orchestrate these processes. While much progress has been made in understanding the regulatory networks governing pattern formation, cell proliferation, and morphogenesis, and while it is clear that they must be integrated, the details surrounding their coordination have not yet been elucidated. Thus, the putative Hairy targets we identified are consistent with known processes involving Hairy and suggest that in addition to regulating pattern formation, Hairy plays a role in transiently repressing other events, perhaps in order to coordinate cell cycle events with the segmentation cascade. Further experiments will be needed to determine how these different roles for Hairy fit together.

Cofactor Recruitment

Corepressor recruitment is an important aspect of transcriptional repression (reviewed in Mannervik et al. 1999; Bone and Roth 2001; Mannervik 2001; Urnov et al. 2001; Jepsen and Rosenfeld 2002). While the sequence-specific DNA-bound repressors contribute to target specificity, the corepressors are thought to help distinguish among particular repression mechanisms to be used via alteration of their recruitment or function. For example, the *Drosophila* devel-

opmental factors Dorsal and T-cell factor (TCF) have been shown to function as either positive or negative regulators of transcription depending on promoter context and cofactor recruitment (Dubnicoff et al. 1997; Cavallo et al. 1998). As each of Hairy's cofactors appears to act differently with Hairy, thereby conferring different developmental consequences, we used the DamID approach, along with polytene chromosome staining, to get our first look at the patterns of Hairy's cofactor recruitment.

The numbers of loci that recruit Groucho, dCtBP, and dSir2 cofactors are consistent with the breadth of interaction they have been shown to exhibit. We identified by DamID profiling 155 loci that recruit Groucho and, as expected, found roughly twice as many sites on polytene chromosomes. Groucho was one of the first corepressors identified and shown to affect a variety of different developmental processes, suggesting that it is a widely used corepressor (Parkhurst 1998; Chen and Courey 2000). In addition to its interaction with Hairy, Groucho was subsequently shown to mediate repression through several other classes of DNA-binding transcriptional regulators including Engrailed, Dorsal, T-cell factor, and Runt (Aronson et al. 1997; Dubnicoff et al. 1997; Jiménez et al. 1997; Cavallo et al. 1998; Roose et al. 1998).

Although Groucho was the first Hairy cofactor identified (Paroush et al. 1994) and its interaction site is often described as Hairy's "major" repression motif (Mannervik 2001), we find that it is associated with only a minority of Hairy targets in Kc cells. Groucho's dominance as a cofactor during segmentation may reflect a preference for Groucho in the reporter assays used previously to assess corepressor activity, or it may be more heavily recruited to Hairy's targets during segmentation. In the future it will be interesting to determine the loci that recruit Groucho in early embryos and, as Groucho binds a number of other repressors, which, if any, of these factors recruits Groucho as its major cofactor.

CtBP was identified more recently, first on the basis of its binding to the C-terminal region of E1A, and in *Drosophila* by its association with the developmental repressors Hairy and Knirps (reviewed in Turner and Crossley 2001; Chinnadurai 2002a). CtBP is an integral component in a variety of multiprotein transcriptional complexes. It has been shown to function as a context-dependent cofactor, having both positive and negative effects on transcriptional repression depending upon the repressor to which it is recruited. More than 40 different repressors have been shown to recruit CtBP. Consistent with this wide recruitment of CtBP, we identified 496 loci that recruit dCtBP by DamID profiling and roughly twice that many sites on polytene chromosomes. A recently reported global protein-protein interaction study showed that the binding partners for Groucho and dCtBP are largely nonoverlapping (Giot et al. 2003). This, along with the near exclusivity of Groucho and dCtBP binding as assayed by DamID and polytene chromosome staining, makes it unlikely that both cofactors work together as a general rule and strengthens the possibility that the binding of each of these factors assembles different protein complexes that are, for the most part, mutually exclusive.

dSir2 was only very recently identified as a corepressor for Hairy and other HES family members (Rosenberg and Parkhurst 2002; Takata and Ishikawa 2003). We identified 107 loci that recruit dSir2 by DamID profiling and roughly

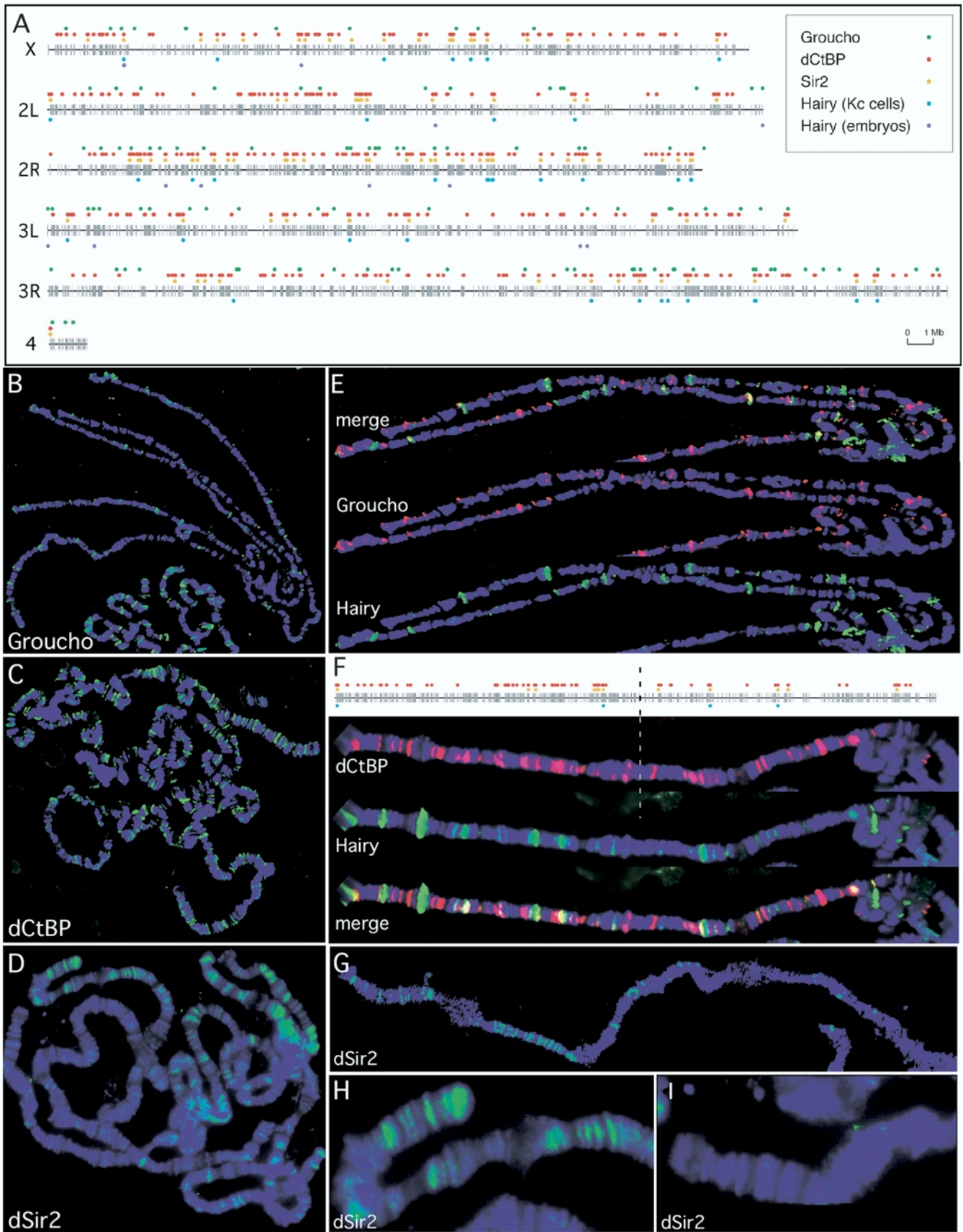


Figure 9. Hairy Shows Context-Dependent Association with Its Cofactors

(A) Sites of Hairy binding and Hairy cofactor recruitment based on DamID. The gray lines depict the relative position on the chromosomes of the approximately 6200 cDNAs on the microarray chip. The blue dots below the line represent Hairy binding sites while the green (Groucho), red (dCtBP), and yellow (dSir2) dots represent the positions of cofactor recruitment.

(B–D) Cofactor recruitment visualized on third instar larval salivary gland chromosomes. Polytene chromosome sets stained (green) with antibodies to Groucho (B), dCtBP (C), and dSir2 (D). All chromosomes were counterstained with DAPI (blue) to visualize the DNA.

(E) Higher magnification view of chromosome arms 2L and 2R costained with Groucho (red) and Hairy (green), and the merged image.

(F) Higher magnification view of chromosome arm 2L costained with dCtBP (red) and Hairy (green), and the merged image, compared to the predicted DamID map. Note that both the DamID projected map and polytene chromosomes have more dCtBP recruitment sites to the left of the dashed line than to the right of the dashed line.

(G) Chromosome arm 3R stained with dSir2 (green), highlighting regional specificity of dSir2 recruitment.

(H and I) Higher magnification view of the distal ends of chromosome arms 2R (H) and 3L (I) from (D), stained with dSir2 (green), showing regional specificity and lack of dSir2 recruitment, respectively.

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twice that many sites on polytene chromosomes. Surprisingly, the distribution of loci recruiting dSir2 identified by DamID profiling, as well as dSir2's staining on polytene chromosomes, shows regional binding specificity (see Figure 9D and 9G). This binding specificity may be a reflection of the different nuclear compartments that these regions of the chromosomes find themselves in (cf. Francastel et al. 2000; Leitch 2000). Sir2 has been described mostly as a protein involved in heterochromatic silencing rather than in euchromatic repression. The number of dSir2 euchromatic sites we observe is similar to that of Groucho, suggesting that euchromatic repressors (in addition to HES family members) are likely to recruit Sir2. Consistent with this, a recent report has described a role for mammalian Sir2 in repressing the muscle cell differentiation program (Fulco et al. 2003). The region-specific binding of dSir2 might reflect a difference in the types of factors it can associate with, or the association of dSir2 with particular chromosomal regions or nuclear domains (cf. Spellman and Rubin 2002).

Interestingly, dCtBP and dSir2 recruitment are largely overlapping, and this association continues outside of those loci where Hairy binds: 90% of dSir2-recruiting loci also recruit dCtBP. dCtBP and dSir2 are unique among transcriptional coregulators in that they both encode NAD⁺-dependent enzymatic activities. As NAD and NADH levels within the cell exist in closely regulated equilibrium (for review see Dang et al. 1997; Ziegler 2000), it is possible that dCtBP and dSir2 function as NAD/NADH redox sensors (cf. Denu 2003; Fjeld et al. 2003). In this way, the cell could use coenzyme metabolites to coordinate the transcriptional activity of differentiation-specific genes with the cellular redox state.

The success of the combination of DamID profiling and polytene chromosome staining results provides a global systematic way in which to address a number of mechanistic questions concerning the rules governing cofactor recruitment. For example, it will be possible to address whether target gene location or promoter structure determines the accessibility of cofactors to specifically bound repressors or whether, conversely, the association of repressors with cofactors influences target gene choice by altering DNA binding specificity. We now have a number of direct Hairy targets and *in vivo* assay systems to use in future experiments addressing questions surrounding Hairy's biological functions and the precise molecular mechanisms it employs to carry out its functions.

Materials and Methods

DamID. To generate Dam–Hairy or Dam–dCtBP, a full-length hairy or dCtBP cDNA fragment was generated by standard PCR using

primers containing a BglII 5' site and a XbaI 3' site, cut with BglII and XbaI, and subcloned into the BglII and XbaI sites of pNMyDam plasmid, as described previously (van Steensel and Henikoff 2000). To generate Dam–Groucho, a full-length *groucho* cDNA fragment (minus the stop codon) was generated by standard PCR using primers containing a BamHI 5' site and a NotI 3' site, cut with BamHI and NotI, and subcloned into the BglII and NotI sites of pCMyDam plasmid, as described previously (van Steensel and Henikoff 2000). Dam–dSir2 was described previously (van Steensel et al. 2001). All four of these constructs are expressed in Kc167 cells (data not shown). Kc cell culture and transfections were performed as described previously (Henikoff et al. 2000). The Kc cells were harvested 24 h posttransfection, then genomic DNA was isolated and processed for microarray hybridizations as described previously (van Steensel et al. 2001).

The UAS–Dam and UAS–Dam–Hairy expression constructs were made by first amplifying the Dam or Dam–Hairy open reading frames by PCR from the appropriate fusion construct described above, then cloning them into the pUASp vector (Rørth 1998) as 5'KpnI–3'XbaI fragments. The resulting UAS–Dam and UAS–Dam–Hairy plasmids (500 µg/ml) were injected along with the pTURBO helper plasmid (100 µg/ml) (Mullins et al. 1989) into isogenic w¹¹¹⁸ flies as described by Spradling (1986). Transgenics were scored by eye color, and the insertions were mapped and balanced using standard genetic methods. These chimeric genes are properly expressed when induced with various Gal4 driver lines (e.g., Engrailed–Gal4; Brand and Perrimon 1993; data not shown). The Dam–Hairy fusion protein is functional because presence of the UAS–Dam–Hairy transgene, but not the UAS–Dam transgene, partially rescues the segmentation phenotype of hairy mutant embryos when induced with an actin–Gal4 driver (rescue is similar to UAS–Hairy; data not shown). As in Kc cells, induced expression of these Dam fusion constructs leads to high levels of nonspecific methylation. Therefore we utilized low-level leaky expression from the minimal promoter of the pUASp vector for these experiments. 2–6-h embryos were collected and dechorionated with 100% bleach. Approximately 500 µl of embryos were crushed in 1 ml of lysis buffer (100 mM Tris [pH 9.0], 100 mM NaCl, 100 mM EDTA, and 5% sucrose). SDS (to 0.5%) and proteinase K (to 100 µg/ml) were added immediately after homogenization, followed by incubation at 55 °C for 2 h. SDS was increased to 1.5%, followed by incubation for an additional 2–3 h. The genomic DNA was isolated and processed for microarray hybridizations essentially as described previously (van Steensel et al. 2001).

Drosophila microarray chips were produced in house (Genomics Shared Resource; Fred Hutchinson Cancer Research Center, Seattle, Washington, United States) for the Northwest Fly Consortium and contain approximately 6200 full-length DGC cDNAs (DGC Release 1; Rubin et al. 2000), as well as approximately 300 clones added by members of the Consortium.

Arrays were scanned using a GenePix 4000 scanner (Axon Instruments, Union City, California, United States), and image analysis was performed using GenePix Pro 3.0. For each array, spot intensity signals were filtered and removed if the values did not exceed 3 standard deviations above the background signal in at least one channel or if the spot was flagged as questionable by the GenePix Pro software. For each spot, background-corrected ratios were natural log transformed and a median-centered normalization strategy was applied across each array. Dam–protein and Dam transfections were independently replicated three times, and the subsequent array comparisons (i.e., Dam–protein/Dam) were analyzed using CyberT (Baldi and Long 2001), a Bayesian t-statistic derived for microarray analysis (<http://genomics.biochem.uci.edu/genex/cybert/>). We employed the default window size of 101 and used a confidence

value of ten in our CyberT analysis. The null hypothesis was rejected and a spot ratio was called significantly changed if $p_{\text{Bon}} \leq 0.05$, where p_{Bon} is the Bayesian p -value adjusted for multiple hypothesis tests using the conservative Bonferroni correction. Based on prior “self versus self” DamID comparisons, we empirically determined a lower-bound $\ln(\text{ratio})$ threshold = $|0.405|$ as an additional significance criterion to discriminate spot intensity signals from the inherent noise in the hybridization process. For each protein analyzed, a fluor-reversed array comparison was performed and used to screen all significant calls for fluor-specific artifacts. For our analyses, we treated the small subset of replicated spots on the array independently. For those cases, both spots were required to be called significant. Reported ratio values were retransformed to \log_2 as a matter of convention. The complete raw and processed datasets can be accessed at <http://www.fhcr.org/labs/parkhurst/supplementary-data/>.

Flies and genetics. Flies were cultured and crossed on yeast-cornmeal-molasses-malt extract medium at 25 °C. The alleles used in this study were the following: h^{7H} *rucal* TM3, h^{12C} *st el* TM3, *Df(3)h¹²²* *Ki roe p¹* TM3, and *prd^{2,3,17}* CyO (D. Ish-Horowitz); FRT82B-P{ γ - Δ 7.2 = PZ} *CtBP³⁴⁶³* γ ⁵⁰⁶ TM3 (N. Perrimon); FRT 82B-*gro^{E47}* TM3 (Phippen et al. 2000); *dSir2^{5,26}*/SM6 and *dSir2^{4,5}*/SM6 (Newman et al. 2002); FRT82B-*ovo^{D1}* TM3, γ *w* hs-FLP22, TM3/CxD, *egh⁷* FM7a (#3902), *ImpL2^{KG0223}* (#14083), *mae^{K06602}* CyO (#10633), *pnt^{A88}* TM3 (#861), and *rgt^{KG03110}* (#13770) (Bloomington *Drosophila* Stock Center, Indiana University, Bloomington, Indiana, United States). Details of these strains are found on FlyBase (<http://flybase.bio.indiana.edu:82/>). *stg^{AR2}* and the *stg-lacZ* reporter lines (pstg β -E2.2, pstg β -E4.9, pstg β -E6.4, pstg β -E6.7) were described previously (Lehman et al. 1999). The genomic locations of the Hairy binding sites in pstg β -E4.9 and pstg β -E6.4 are 25072653 and 25080219, respectively. Germline clones for *dCtBP* and *groucho* were generated as previously described (Poortinga et al. 1998, Phippen et al. 2000). The pstg- β E4.9^{Hairy} transgenic flies were generated by injecting vector (500 μ g/ml) along with the pTURBO helper plasmid (100 μ g/ml) (Mullins et al. 1989) into isogenic w^{1118} flies as described by Spradling (1986). Transgenics were scored by eye color, and the insertions were mapped and balanced using standard genetic methods.

Embryo analysis. Larval cuticle preparations were prepared and analyzed as described by Wieschaus and Nüsslein-Volhard (1986). Immunohistochemical detection of proteins in embryos was performed as described previously (Parkhurst et al. 1990) using Alkaline Phosphatase-coupled secondary antibodies (Jackson Laboratory, Bar Harbor, Maine, United States) visualized with Substrate Kit II reagents (Vector Laboratories, Burlingame, California, United States). Antisera used were as follows: antiMyc (9e10, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, California, United States).

Immunohistochemical whole mount RNA in situ hybridization was performed according to the protocol of Tautz and Pfeifle (1989). Digoxigenin-substituted probes were obtained by PCR amplification with primers to the vector just 3' of the cDNA insert.

EMSA. EMSA was carried out using either bacterially expressed GST or GST-Hairy (full-length) proteins, similar to the procedure described by Van Doren et al. (1994) and Rosenberg and Parkhurst (2002). Briefly, 40 fmol of ³²P-end-labeled probe of each oligo was incubated with either GST- or GST-Hairy-purified proteins (200 ng each), in a 25- μ l reaction supplemented with binding buffer (5% glycerol, 20 mM HEPES [pH 7.6], 50 mM KCl, 1 mM EDTA, 1 mM DTT, and 10 ng/ μ l poly dI-dC) at room temperature. Where indicated, the binding was performed in the presence of 15-fold excess of unlabeled wild-type or mutated *ac* competitor oligos. Following incubation, the complexes were resolved using 0.5% TBE-PAGE gels and visualized by autoradiograms. The following oligos were used (forward primers are shown): *ac* 5'-TAAACCGTGGCAGCCGG-CACGCGACAGGGCCAGGTTTT-3'; *egh* *egh1* 5'-TGCG-CGTCCAGCGCGTTC-3', *egh2* 5'-TCATTCGACGCGGAATCT-3', and *egh3* 5'-GCCGGACACGGATGATGG-3'; mutated *ac* oligo 5'-TAAACCGTGGCAGCCGGCAGCGACAGGGCCAGGTTTT-3'; mutated *stg* oligo 5'-TCTACCACACAAAACCTCGCACGC-GAAAACGGG-3'; *prd* 5'-AAGTGACACGGCTCCCG-3'; and *stg* 5'-AAACACACGCGCGGAAA-3'.

Hairy binding site bioinformatics analysis. Several bioinformatics approaches were employed to analyze Hairy target gene promoters. In particular, *Drosophila* promoter sequences were captured using Apollo Genome Sequence and Annotation Tool (Lewis et al. 2002). Match v1.0-public (BIOBASE Biological Databases, Wolfenbüttel, Germany) was used to search promoter sequences for known transcription factor binding sites using a library of mononucleotide-weighted matrices from TRANSFAC v6.0. Match v1.0-public employs the core- and matrices-matching algorithms published by

Quandt et al. (1995). Sequences were interrogated using only high-quality *Drosophila* transcription factor binding sites found in TRANSFAC v6.0, and the software parameters were adjusted to minimize the sum of false positives and false negatives. The number of Hairy binding sites found in target gene promoters was tallied (excluding “hits” to AT-rich regions [assigned to CF2-II, BRC-Z1, and BRC-Z4] that were ubiquitous in both the target and nontarget sequence under analysis). Using the Hairy site closest to transcription start site, the composition of transcription factor binding sites adjacent to (within 500 bp of) the Hairy site was assessed. This was also performed for non-Hairy targets selected because they contained one or more core C-box sequences. Matrices were compared that matched percentages of known Hairy targets (*egh2*, *egh3*, *prd1*, *ac1*, and *stg1*) to C-box-containing nontargets.

Chromosomes. Wild-type or pstg β E4.9 third instar larval salivary gland polytene chromosomes were prepared and stained for endogenous proteins essentially as described by Andrew and Scott (1994). Antisera used were as follows: rat anti-Hairy polyclonal (1:50 dilution; gift of J. Reinitz; Kosman et al. 1998), mouse anti-Groucho monoclonal (1:40 dilution; gift of C. Delidakis; Delidakis et al. 1991), mouse anti-dCtBP polyclonal (1:100; Poortinga et al. 1998); mouse anti-dSir2 polyclonal (1:20 dilution; Rosenberg and Parkhurst 2002); rabbit anti- β -galactosidase polyclonal (1:1000); donkey antirat Alexa 488 (1:1000 dilution; Molecular Probes, Eugene, Oregon, United States); and goat antimouse Texas Red (1:200; Jackson Immuno-Research Laboratories, West Grove, Pennsylvania, United States). Chromosomes were viewed on an Olympus (Tokyo, Japan) IX-70 inverted microscope equipped with a 40 \times /N.A. 1.35 oil immersion objective. Three-dimensional stacks were collected using the Delta-Vision softWoRx acquisition software (Applied Precision, Issaquah, Washington, United States), and out-of-focus information was removed using a constrained iterative deconvolution algorithm (Agard et al. 1989).

The insertion site for the pstg β -E4.9 reporter line was performed as described by Pardue and Gall (1975) using DIG-substituted probes according to the protocol of Tautz and Pfeifle (1989).

Supporting Information

Dataset S1. Complete List of Binding Loci for Hairy in Kc Cells and Embryos As Well As the Cofactors Groucho (Kc Cells), dCtBP (Kc Cells), and dSir2 (Kc Cells)

Found at DOI: 10.1371/journal.pbio.002017sd001 (2.9 MB XLS).

Dataset S2. DamID Primary Binding Data for Hairy in Kc Cells

Found at DOI: 10.1371/journal.pbio.002017.sd002 (11.8 MB XLS).

Dataset S3. DamID Primary Binding Data for Hairy in Embryos

Found at DOI: 10.1371/journal.pbio.002017.sd003 (11.8 MB XLS).

Dataset S4. DamID Primary Binding Data for Groucho in Kc Cells

Found at DOI: 10.1371/journal.pbio.002017.sd004 (11.8 MB XLS).

Dataset S5. DamID Primary Binding Data for dCtBP in Kc Cells

Found at DOI: 10.1371/journal.pbio.002017.sd005 (11.8 MB XLS)

Dataset S6. DamID Primary Binding Data for dSir2 in Kc Cells

Found at DOI: 10.1371/journal.pbio.002017.sd006 (11.8 MB XLS)

Dataset S7. List of the 155 Target Loci That Recruit Groucho (Duplicates Removed)

Found at DOI: 10.1371/journal.pbio.002017.sd007 (230 KB XLS).

Dataset S8. List of the 496 Target Loci That Recruit dCtBP (Duplicates Removed)

Found at DOI: 10.1371/journal.pbio.002017.sd008 (276 KB XLS).

Dataset S9. List of the 107 Target Loci That Recruit dSir2 (Duplicates Removed)

Found at DOI: 10.1371/journal.pbio.002017.sd009 (44 KB XLS).

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Conflicts of interest. The authors have declared that no conflicts of interest exist.

Author contributions. DB, AO, and SMP conceived and designed the experiments. DB, AO, JJD, JV, AER, and SMP performed the experiments. DB, AO, JJD, JV, AER, and SMP analyzed the data. SMP wrote the paper. ■

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