Hedgehog targets in the *Drosophila* embryo and the mechanisms that generate tissue-specific outputs of Hedgehog signaling

Brian Biehs, Katerina Kechris*, SongMei Liu and Thomas B. Kornberg†

**SUMMARY**
Paracrine Hedgehog (Hh) signaling regulates growth and patterning in many *Drosophila* organs. We mapped chromatin binding sites for Cubitus interruptus (Ci), the transcription factor that mediates outputs of Hh signal transduction, and we analyzed transcription profiles of control and mutant embryos to identify genes that are regulated by Hh. Putative targets that we identified included several Hh pathway components, mostly previously identified targets, and many targets that are novel. Every Hh target we analyzed that is not a pathway component appeared to be regulated by Hh in a tissue-specific manner; analysis of expression patterns of pathway components and target genes provided evidence of autocrine Hh signaling in the optic primordium of the embryo. We present evidence that tissue specificity of Hh targets depends on transcription factors that are Hh-independent, suggesting that ‘pre-patterns’ of transcription factors partner with Ci to make Hh-dependent gene expression position specific.

**KEY WORDS:** *Drosophila*, Hedgehog, Cubitus interruptus

**INTRODUCTION**
Hedgehog (Hh) is a secreted signaling protein that regulates the growth and patterning of many organs. First identified because of its roles in *Drosophila* embryonic and imaginal disc development, it is now understood to be essential to most organs in *Drosophila* and higher vertebrates. Despite its systemic importance, Hh signaling is local and is dependent upon Hh that is expressed and secreted by discrete sets of cells in each tissue that it regulates. In each context, organ-specific programs of gene expression, morphogenesis and cell-cycle regulation depend upon Hh regulation. The mechanism that endows Hh signaling with tissue specificity has not been fully elucidated.

Hh signaling has been most thoroughly analyzed in the *Drosophila* wing imaginal disc, where Hh is expressed in posterior compartment cells. Signaling to adjacent anterior cells begins when Hh engages the participation of two membrane proteins in target cells: Patched (Ptc) and Smoothened (Smo). Hh activates Smo (Stone et al., 1996; Taipale et al., 2002) and initiates the transformation of a complex of proteins that includes an inactive form of the transcription factor Ci (Jia et al., 2003; Lum et al., 2003; Ruel et al., 2003). Ci mediates most, and perhaps all, of the output of the Hh pathway (Alexandre et al., 1996; Méthot and Basler, 2000). Although support for the latter might come from linked cis-regulatory elements or participation of co-factors (Müller and Basler, 2000), we do not yet know whether such shared access is a feature of all Ci binding sites and, if it is, how the distinct activator and repressor functions are realized in the context of their common sites. Possible mechanisms include differential affinity, input from linked cis-regulatory elements or participation of co-factors (Müller and Basler, 2000). Although support for the latter might come from the observation that full activation of the *dpp* heldout enhancer requires both Hh signaling and the wing ‘selector’ protein Vestigial (Hepker et al., 1999), the mechanisms that determine tissue-specific activation of other Hh targets remain unidentified. Recent studies that used chromatin binding to identify target sequences for mouse Gli1 and Gli3 proteins in neural tissues and whole limb buds,
respectively (Vokes et al., 2007; Vokes et al., 2008), identified many novel GlI-responsive cis-regulatory elements. Characterization of these elements led to the conclusion that the GlI1 activator and the GlI3 repressor recognize and regulate common sequences.

The number of known Hh targets in *Drosophila* is small. The work described here was undertaken to obtain a better understanding of targets and to investigate the mechanism of Ci action and specificity. We employed expression array assays to identify genes that have Hh-dependent expression in embryos, and chromatin-binding assays to identify genes linked to genomic regions that are recognized by CiAct and CiRep. Putative targets were selected that bind both Ci forms and are Hh-dependent. Characterization of several novel targets highlighted two key aspects of Hh signaling. With the exception of several genes that encode core components of the Hh signal transduction pathway, all targets are expressed in restricted domains within Hh-responsive tissues. For *Ecdysone-inducible gene L2 (ImpL2)*, a novel Hh-regulated gene that is expressed in the tracheal primordium, we show that expression is dependent upon the transcription factor Tracheless (Trh), expression of which is not dependent upon Hh, and that ImpL2 expression is restricted to only a portion of the cells that express Trh. Expression of this Hh target is, therefore, specified by the combined activities of Hh-dependent Ci and Hh-independent Trh.

**MATERIALS AND METHODS**

*Fly stocks and crosses*

Homozygous null mutant embryos lacking *ptc* or *hh* function were generated from the following alleles: *hh*(*1C*), *hh*(*1C*), *ptc*(*08*) and *ptc*(*1C*). *ci*-null embryos were generated from a cross of *ciRES* (Méthot and Basler, 1999) in a *ci*-null background (ci(*4l/4l*), ci-m1-m4. *smo* germline clones were generated from a cross of *y w hs-FLP*<sup>129</sup>; *smo<sup>0</sup> FRT40A/Cyo* females to *+/y, Ovo<sup>3P</sup>, FRT40A/Cyo* males (Chou and Perrimon, 1996). Embryos and first instar progeny were heat shocked daily at 37°C for 1 hour prior to eclosion. *UAS-C<sup>ptcm4</sup>-Gal4 (CiAct)* was obtained from S. Smolik (Oregon Health and Science University, Portland, OR, USA) and the *Dam* alone transgenic line was obtained from S. Parkhurst (Fred Hutchinson Cancer Research Center, Seattle, WA, USA).

*In situ hybridization and immunostaining*

Digoxigenin-labeled anti-sense RNA probes were hybridized to whole-mount embryos (*O’Neill and Bier, 1994*). Antibodies used for immunostaining were: rat anti-2A1 (Ci<sup>P</sup>; R. Holmgren, Northwestern University, Evanston, IL, USA; 1:200), mouse anti-Produced (I. Guerrero, Universidad Autonoma de Madrid, Madrid, Spain; 1:200), mouse anti-Phelan (B. van Steensel, Netherlands Cancer Institute, Amsterdam, The Netherlands). *DamCi* fusion cassettes were subcloned into puAST.

*DamID constructs*

N-terminally fused DamID constructs were generated by PCR amplification of *BagI/II-XbaI* fragments of *Ci<sub>T7a* (Aza-Blanc et al., 1997) and *Ci<sub>ptcm4</sub>-ned* (S. Smolik) using the forward primer 5’-TAAGATCTTATAGCACTCAGGATCTATAG3’ and reverse primers 5’-Taatctc-AGAGTCTGCACTCAGTCTATAG3’ for *Ci<sub>T7a*</sub> and 5’-Taatctc-AGAGTCTGCACTCAGTCTATAG3’ for *Ci<sub>ptcm4</sub>-ned*. PCR products were digested with *BagI/II* and *XbaI* and ligated to pNDamMyc (B. van Steensel, Netherlands Cancer Institute, Amsterdam, The Netherlands). DamCI fusion cassettes were subcloned into puAST.

*DamID analysis*

DamID probes were prepared following the protocol described at [http://research.nki.nl/vansteenselab/damid.htm](http://research.nki.nl/vansteenselab/damid.htm). Genomic DNA was isolated from 2- to 6-hour-old embryos containing DamCI or Dam transgenes. DNA (2.5 µg) was ethanol-precipitated, digested with *DpnI* and ligated to annealed adaptor oligos for 2 hours at 16°C (5’-CTAT- TACGACTCACTATAGGGCCAGCGTGTCGCCGAGGGA-3’ and 5’-TCTCGGCCGCG-3’). After heat inactivation, the ligation mixture was digested with *DpnII*, which cuts unmethylated DNA. PCR amplification of Dam-methylated DNA was carried out using the PCR Advantage Mix (Clontech) and the primer 5’-GGTCCGGCCCGAGGATGAC-3’. Labeling and hybridizations were performed by NimbleGen Systems (Reykjavik, Iceland). The amplified methylated fragments were labeled with Cy3 or Cy5 in a dye-swap configuration to eliminate bias and were hybridized to NimbleGen Systems’ whole genome tiling arrays consisting of 375,000 60mer probes spaced ~300 bp apart (Choksi et al., 2006). Three replicates of control (Dam alone) and experimental (DamCi<sub>4a</sub> and DamCi<sub>4b</sub>) samples were analyzed. Raw intensity data were analyzed as described below.

All analyses were performed in R v2.7.1 (R Development Core Team, [http://www.R-project.org/](http://www.R-project.org/)) unless otherwise stated. At each tiling array feature, log ratios between the experimental and control samples were calculated and normalized using ‘limma’ (Smyth, 2005), applying ‘loess’ and ‘cubic spline’ for normalization within arrays and between arrays, respectively (Wormald et al., 2006). To predict binding regions, each feature was tested for significant positive intensity using a one-sided t-test. Because sample size was small (*n=3*), information was pooled from all features to obtain more stable variance estimates for the t-statistic using the limma package. To identify genomic regions of high signal intensity, *P*-values for consecutive features were ‘averaged’ using meta-analysis based on combining *P*-values (for details, see Kechristis et al., 2010). Because of the large number of significance tests, *P*-values were corrected for false discovery rate (FDR) control (Benjamini et al., 2001) with window size set at four. Binding regions were created by scanning features as they are ordered along each chromosome. If a feature had an adjusted *P*-value below the set FDR cutoff (0.02 for Dam<sup>ptcm4</sup>; 0.001 for DamCi<sub>4a</sub>), a new binding region was formed. The next feature passing the cutoff in the linear chromosome was then evaluated. This procedure was continued in a stepwise fashion.

*Overlap of DamID protected regions*

The overlap binding regions were evaluated using two metrics: (1) overlap of set of all proximal transcripts and (2) base pair overlap between binding regions. To compare these values with chance occurrence, binding regions for Ci<sub>4b</sub> were generated randomly fifty times based on length distribution and number of binding regions. Each random set of binding regions was compared with the Ci<sub>4a</sub> binding region, and the two overlap metrics were calculated. To obtain gene and base pair overlap, enrichment scores of original values for overlap metrics were divided by values based on an average of 50 randomizations. This analysis was also repeated by performing the binding region randomizations on each of the 21 transcription factors analyzed in a previous study (MacArthur et al., 2009) and comparing those with the Ci<sub>4a</sub> binding regions. We found extensive overlap to the same regions identified by MacArthur and colleagues. Given the extensive overlap within the MacArthur data set for the many different transcription factors that they and others have profiled, these binding sites appear to be detected by several contrasting techniques and are of uncertain significance.

*Incidence and frequency of Ci motifs*

The sequences of the DamID binding regions were extracted from FlyBase v4.0, and occurrence of TGGGTGGTC and reverse complement were counted (Perl script v5.10.0). The rate of this motif, as well as the occurrences of ‘relaxed consensus’ sequences, in binding regions and genome were calculated by dividing counts of motif by the total length of binding regions or genome, respectively. Enrichment was the rate of motif occurrences in the peak set divided by the rate in the genome. *P*-values for enrichment assumed a Poisson distribution. The strict consensus (TGGGTGGTC) occurs 2.5 and 2.4 times more frequently in CiAct and CiRep binding sites, respectively, than would be expected by chance (*P*<0.001 and 7.2<10<sup>-8</sup>, respectively). The relaxed degenerate Ci motif (YGSGDGNN) occurs 1.2 and 1.3 times more frequently in Ci<sub>4a</sub> and Ci<sub>4b</sub> binding sites, respectively.
RESULTS

Specificity of Ci binding to the Drosophila genome

We used the DamID technique (van Steensel et al., 2001) to locate regions bound by Ci in the Drosophila genome. This method directs DNA methylation to sites of sequence-specific binding by expressing a fusion protein composed of a transcription factor and DNA adenine methyltransferase (Dam), and employs hybridization to DNA microarrays to detect genomic fragments for which methylation confers protection from endonuclease digestion. We generated two DamCi fusion proteins: a constitutive CiAct of Dam (DamCiAct) fused to the N terminus of a Ci mutant that activates Hh targets independently of Hh signaling (Chen et al., 1999), and Dam fused to the N terminus of Ci76 (DamCiRep) (Aza-Blanc et al., 1997). Examination of wing phenotypes after expression at high levels in wing discs (MS1096GAL4) revealed that CiAct and CiRep functionality was preserved in the respective fusion proteins (not shown).

To identify Ci targets, methylated DNA fragments were collected from stage 10-11 embryos expressing DamCiAct or DamCiRep. At this stage, organogenesis is active and Hh signaling activity is required in many areas. Three replicates were analyzed for each construct; Dam alone embryos served as a control for non-specific nuclease protection but simply assume that the location and size of targets analyzed the genome in the vicinity of regions bound by DamCiAct (see Table S1 in the supplementary material). Median protected region length was 2133 bp for DamCiAct and 993 bp for DamCiRep. The differences in frequency and length might reflect differences in biological activity of the CiAct and CiRep constructs, different levels of expression, different affinities or other factors, but, irrespective of cause, we assume that this method has a high false positive rate (see Materials and methods for further discussion), as do other related techniques that monitor chromatin binding (Yokes et al., 2008; Zeitlinger et al., 2007). To establish the criteria that distinguish functional sites from false positives, we assessed four properties: (1) common binding by CiAct and CiRep, (2) presence of Ci binding motifs, (3) linkage to known Hh targets and (4) Hh dependence of expression levels of linked genes.

Available evidence indicates that CiAct and CiRep can bind to and function at common sites (Müller and Basler, 2000), and a significant number of the DamID-protected regions are shared. 729 out of 2438 (30%) of the DamCiAct-protected regions overlapped with DamCiRep-protected regions, with a median overlap of 1231 bases; 75% of the overlaps were between 383 and 2147 bases. The base pairs that overlapped were 8.6 times the number expected by chance. We do not understand the basis for extensive regions of nuclease protection but simply assume that the location and size of each region correlates in some way with the presence, number and affinity of binding sites, and perhaps with local chromatin conformation. Irrespective of the underlying mechanism, we analyzed the genome in the vicinity of regions bound by DamCiAct or DamCiRep to identify transcription units that contain or have adjacent upstream or downstream binding sites for both. The number of transcripts found that contain or are proximal to both DamCiAct and DamCiRep binding regions was 2108. The correlation of the transcripts that are associated with both binding regions was

Expression array analysis

RNA was isolated, amplified and labeled as described previously (Klebes and Kornberg, 2008). Data were processed with Cluster and Treeview (Eisen et al., 1998), and GenePix PRO (Axon Instruments). Normalization for cluster analysis was carried out with NOMAD 2.0 (University of California, San Francisco, USA). Genes chosen had combined median intensity of >300 above background (both channels) in >80% of experiments, and above a threshold of 1.4 (0.55 of the log2-transformed ratios). The fold-change and cluster selection procedure identified 147 transcripts. Using a post hoc FDR threshold of 1.4 (0.55 of the log2-transformed ratios). The fold-change and concordance percentages were higher between replicates within the same group and were lower between different groups (see Table S3 in the supplementary material). For example, the correlation was 0.62 between replicates of the kk arrays whereas the median correlation with other arrays was 0.2.

Data access

Data for the DamID and expression arrays are in the GEO database. Results from DamID are the normalized log2-transformed ratios of DamCi/DamAlone signals. The data include three biological replicates for both DamCiAct / DamAlone (GSE23999) and DamCiRep / DamAlone (GSE24024). The gene expression array data (GSE24038) are both the log2-transformed ratios and fluorescent intensities for 14 experiments comparing expression in mutant embryos with control siblings. GSE24055 includes all of the deposited data as one superseries.

De novo motif analysis

In addition to word searches of the Ci motifs, two programs were used to query the peaks associated with the 52 high confidence genes. All peaks neighboring the 52 genes (91 peaks, for a total of 504,561 bp) were extracted and masked for repeats using the RepeatMasker tool (http://www.repeatmasker.org/) that identifies interspersed repeats and low complexity DNA sequences. The masked sequences were then scanned for repeating patterns using default options for the MEME software (http://meme.sdsc.edu) and the oligo-analysis tool at the RSA Tools website (http://rsat.ulb.ac.be/rsat). MEME found some local repeats (e.g. CACACACA) despite repeat masking. The RSA analysis was more successful: of the 35 most significant oligonucleotide sequences that were over-represented (occurrence E-value<10–8), most could be clustered by similarity using the RSA pattern assembly tool into seven motifs of at least three sequences. One included a portion of the Ci consensus site TGGTGGT. Other motifs included GTCTCGC, TGCTGCTC, GTCTTT, CGGCCGC, TTGTGT and TATTTA. We cannot distinguish whether these sequences are variations of the Ci consensus site or are alternative regulatory sequences. Otherwise, these searches, in general, identified little of note.

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To assess the degree of similarity in expression values for array replicates, Pearson’s correlation coefficient and a concordance percentage were calculated between each pair of replicates within a group and between replicates across groups. Missing values were removed from both analyses. Higher correlations (positive) or concordance percentages, with a maximum of 1.0 and 100%, respectively, indicate that the arrays are generally in agreement. Although there was some variability for replicates within a group (e.g. for the Smo group, one of the lowest correlation values was 0.3 between two of the replicates), the median correlation and concordance percentage were higher between replicates within the same group and were lower between different groups (see Table S3 in the supplementary material). For example, the correlation was 0.62 between replicates of the kk arrays whereas the median correlation with other arrays was 0.2.

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statistically significant (1.4× that expected by chance; P-value <2.2×10−6, Fisher’s test) and they represented 67% of DamCIRep (2108 out of 3160) and 54% of DamCIAct (2108 out of 3939) binding region-associated transcripts.

We examined the DamCIRep peaks to locate sequences that conform to known Ci binding preferences. Previous work defined a consensus binding sequence for the Gli and Ci proteins (TGGTGGTC) (Hallikas and Taipale, 2006; Kinzler and Vogelstein, 1990; Pavletich and Pabo, 1993), as well as variants at human ptc (PTCH1 – Human Gene Nomenclature database) (Kinzler and Vogelstein, 1990), Drosophila wg (Von Ohlen et al., 1997), Drosophila dpp (Müller and Basler, 2000) and mouse Foxa2 (Sasaki et al., 1997). Probing the Drosophila genome for these sequences (YGSGTGGHC and HDSSHGVHS) identified many sites, but their incidence in the DamID peaks was not significantly above that expected by chance (see Materials and methods). By probing with the consensus sequence (TGGTGGTC), which represents an unknown fraction of Ci binding targets, we found that its frequency in the DamCIAct and DamCIRep peaks was 2.4- to 2.5-fold greater than expected by chance. Most consensus sequence motifs were present in one copy only (e.g. motifs in 89% of DamCIAct peaks were singles) and their proximity to transcription start sites was without apparent bias. We conclude that our current understanding of Ci binding specificity leaves us without the means to distinguish between bona fide binding sites and background, but that these statistics, as well as the correlation with known target genes described in the following section, suggest that the presence of consensus sequence motifs is a positive indicator for functional binding.

Next, we asked if known targets of Hh signaling were among the 2108 genes linked to DamCIAct and DamCIRep peaks. Binding regions for DamCIAct and DamCIRep were detected within 7 kb of the transcription units of 27 out of 30 known targets (Table 1). Plots of DamID protection at five representative targets, ptc, wg, rdx, bagpipe (bap) and atonal (ato), are shown in Fig. 1. At ptc, a 35 kb DamID-protected region included most of the transcription unit as well as ~20 kb upstream. Six Ci consensus binding sites were in the protected region; the sites in the upstream sequence have been shown to have Hh-responsive enhancer activity (Alexandre et al., 1996; Forbes et al., 1993). At wg, a 2.5 kb element upstream of the transcription start site drives expression in metameric stripes (Lessing and Nusse, 1998). This region has sequences that match the degenerate consensus Ci binding site (see Table S2 in the supplementary material), and both DamCI forms conferred nuclease protection. rdx requires Hh activity for expression (Kent et al., 2006), but its regulatory sequences have not been characterized. DamCI analysis revealed binding by both DamCI forms within rdx. Enhancers responsible for Hh regulation of ato
expression in eye discs and the PNS of the embryo have been identified (Sun et al., 1998). The DamID-protected regions at ato spanned the identified enhancer elements. We interpret the consistency of DamCi\textsuperscript{Act} and DamCi\textsuperscript{Rep} binding, the presence of Ci consensus binding sites in DamCi protected regions and the tight correlation of DamID signal to known Hh targets as evidence that this DamID analysis detected Ci binding sites that function in vivo.

**Hedgehog responsive genes**

To identify Hh-dependent transcripts, RNA extracted from five types of stage 10-11 mutant embryos was compared with controls by hybridization to whole genome expression arrays. Mutant embryos for three positive regulators of the pathway (Hh, Smo and Ci) and one negative regulator (Ptc) were tested, as well as embryos in which Ci\textsuperscript{Act} was ectopically expressed (daGal4\textsuperscript{+/-} Ci\textsuperscript{Act}). Clustering genes according to similarity of responses yielded a group of 147 (Fig. 2A). The abundance of transcripts from these 147 genes was decreased in embryos with compromised Hh signaling (hh, smo, ci), and increased in embryos with elevated Hh signaling (ptc, daGal4\textsuperscript{+/-} Ci\textsuperscript{Act}). Mutants lacking the function of Hh pathway components developed similar cuticular phenotypes, therefore, this expression array analysis shows that the cuticular phenotypes have a common mechanistic basis in a failure to regulate the Hh signaling pathway and Hh target genes.

Genes in this group of 147 might be either direct or indirect targets, and as wg is a direct target at each segment border of the embryo, many of these Hh-responsive genes could be direct targets of Wg rather than Hh. Ci binding is presumably a property that distinguishes Hh targets, and 52 genes of the 147 had regions within, or adjacent to, their transcription units that were protected by DamCi\textsuperscript{Act} or DamCi\textsuperscript{Rep}. Published work has shown that Hh regulates 13 of the 30 known Hh targets in embryos, whereas the others are targeted at later stages or are negatively regulated (Table 1). Five of the thirteen embryo targets (atodal, bagpipe, engrailed, rdx and wg) are among the 52 candidate genes. Of the seven that are not, three (Drop, Wnt4, rhomboid) responded as expected of Hh targets in some arrays but their responses in other arrays were below our thresholds; four (ptc, huckebein, seven up, stripe) were not included in the cluster analysis because their transcripts were not represented in the hybridization probes (presumably for failure to amplify, meaning that only nine of the known embryo targets were queried in this analysis); and one (lethal of scute) is expressed in only six cells per hemi-segment and the levels of its transcripts might have been below detection. We suggest that the combination of Ci binding sites revealed by DamID analysis, together with the response to Hh signaling revealed by the expression array analysis, identifies a set of 52 probable Ci targets. In the following sections, we describe our characterization of some of these genes as well as several others that are co-regulated.

**Tissue-specific responses to Ci**

ptc and rdx are targets of Hh signaling that encode components of the Hh signal transduction pathway, and available evidence indicates that both are expressed in all cells that respond to Hh. By contrast, expression of all other Hh targets appears to be tissue-specific. Thirty known targets listed in Table 1 do not encode components of Hh signal transduction, and each has been shown to be expressed in tissue-specific patterns. Our analysis of many of the new candidate targets indicates that they are also expressed in only a subset of Hh-responsive tissues. Our analysis of candidate target genes expressed in the embryo dorsal ectoderm, visual primordia and tracheal placodes is described below.

**Dorsal ectoderm**

Among the genes that are expressed in segmentally repeated stripes in the embryo, three are members of the odd skipped family. These genes, drumstick (drm), sister of odd and bowl (sob) and odd skipped (odd), are linked on chromosome 2L and are associated with three regions of overlapping DamCi\textsuperscript{Act} and DamCi\textsuperscript{Rep} binding (Fig. 3A). Although drm is the only candidate that fulfills both the DamCi binding and cluster analysis criteria, odd and sob expression changed in ways that are consistent with regulation by Hh signaling. drm, odd and sob were expressed in nearly identical patterns in stage 11 embryos (Fig. 3C-E). The stripes of drm, sob and odd expression were adjacent and posterior to hh-expressing cells in each parasegment (Fig. 3C; data not shown), and expression in these domains is consistent with regulation by Hh. We compared drm, sob and odd transcription in embryos that express Ci\textsuperscript{Act} ubiquitously (daGal4\textsuperscript{+/-} Ci\textsuperscript{Act}) with controls using ptc expression as reference. In stage 11 embryos, ptc was expressed in many places, whereas in the trunk ectoderm, expression was confined to two ectodermal stripes in each parasegment, one to
Table 1. Hh target genes

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<td>sloppy paired 1*</td>
<td>ladybird early</td>
<td>↓</td>
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<td>Jagla et al., 1997</td>
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<td>sloppy paired 2*</td>
<td>ladybird late</td>
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<td>snail*</td>
<td>lethal of scute</td>
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<td>Bossing and Brand, 2006</td>
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<tr>
<td>warniu*</td>
<td>seven up</td>
<td>↑</td>
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<td>Bronzielli et al., 2002</td>
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<td></td>
<td>stripe</td>
<td>↑</td>
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<td>Piepenburg et al., 2000</td>
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Cell cycle regulators

| Cap-G*               | CycE*                 | ↑      | ↑      |     | Duman-Scheel et al., 2002 |
| CycA*                | CycD                  | ↑      | ↑      |     | Duman-Scheel et al., 2002 |
| miranda*             |                       |       |       |     |                       |
| Rac2*                | sticky*               |       |       |     |                       |

Signaling pathways

| fat*                 | dpp                   | ↑      | ↑      |     | Dominguez et al., 1996 |
| naked cuticle*       | hedgehog*             | ↑      | ↑      |     | Heberlein et al., 1995 |
| Toll-6*              | ptc                   | ↑      | ↑      |     | Alexandre et al., 1996 |
|                      | roadkill*             | ↑      | ↑      |     | Kent et al., 2006 |
|                      | rhomboid              | ↑      | ↑      |     | Alexandre et al., 1996 |
|                      | Serrate               | ↓      | ↓      |     | Alexandre et al., 1996 |
|                      | vein                  | ↓      | ↓      |     | Amin et al., 1999 |
|                      | wingless*             | ↑      | ↑      |     | Von Ohlen et al., 1997 |
|                      | Wnt4                  | ↑      | ↑      |     | Buratovich, 2000 |

Other and unknown

| CG6850*              | Cad99C                | ↑      | ↑      |     | Schlichting et al., 2005 |
| CG1210*              | Cad86C                | ↑      | ↑      |     | Schlichting et al., 2005 |
| CG3424*              | eyes absent           | ↑      | ↑      |     | Pappu et al., 2003 |
| CG4444*              | pxb                   | ↓      | ↓      |     | Inaki et al., 2002 |
| CG10176*             |                       |       |       |     |                       |
| CG10641*             |                       |       |       |     |                       |
| CG16815*             |                       |       |       |     |                       |
| CG8589*              |                       |       |       |     |                       |
| CG8414*              |                       |       |       |     |                       |
| CG13366*             |                       |       |       |     |                       |
| CG1677*              |                       |       |       |     |                       |
| CG6398*              |                       |       |       |     |                       |
| CG12702*             |                       |       |       |     |                       |
| CG8965*              |                       |       |       |     |                       |
| CG15628*             | Mes4*                 |       |       |     |                       |

Transcriptional or translational regulation

| bancal hnRNP*         |                       |       |       |     |                       |
| cactus*               |                       |       |       |     |                       |
| domino DNA dependent ATPase* |               |       |       |     |                       |
| mRNA capping enzyme*  |                       |       |       |     |                       |
| Hrb27C*               |                       |       |       |     |                       |
| trailer hitch*        |                       |       |       |     |                       |

The current study identified 52 genes (*) based on two criteria: linkage with regions protected by DamCiAct and DamCiRep; and array clustering (Fig. 2A). These 52 genes, as well as previously identified targets (column 2), are listed and organized according to molecular function. For previously identified genes, published data regarding up (+) or down (−) regulation by Hh signaling or Ci activity and Hh- or Ci-dependence of expression in the embryo (+) are summarized in the adjacent columns. All previously identified genes are linked with DamCi-protected sequences, except for CycD, Cad99C and Cad86C. Hh-dep, regulation by Hedgehog signaling; Ci-dep, regulation by Cubitus interruptus activity; Emb, dependence on Hh or Ci in the embryo; DamCi, genes linked with DamCi-protected sequences.
either side of each hh stripe (Hidalgo and Ingham, 1990). Levels of expression were higher in the more posterior stripe of each pair, and with ubiquitous Ci\textsuperscript{Act}, the general level of ptc expression increased; the more intensely expressing posterior stripe broadened posteriorly, and the more anterior stripe broadened anteriorly. These changes are consistent with expansion of ptc expression to all cells except for those that express En. drm, sob and odd each responded to ubiquitous Ci\textsuperscript{Act} with posterior expansion of each stripe by two to three cells (Fig. 3B-I). In wild-type germband-extended embryos, drm and sob were also expressed in the presumptive hindgut and odd was expressed in the presumptive foregut. We did not observe changes to these domains of expression after ubiquitous expression of Ci\textsuperscript{Act}, nor did we observe ectopic expression in any other tissues in which Hh signaling is also active.

Visual primordium
The visual primordium has anterior and posterior optic lobes (AOL and POL, respectively), and contains the precursor cells for Bolwig’s organ, the larval eye. During stage 11, the placode adopts a V-like shape and has conspicuous anterior and posterior lips. hh is expressed in the visual primordium and its expression pattern changes rapidly as the embryo matures. In situ hybridization and enhancer trap expression revealed that, at early stage 11, hh expression extended from the POL into the ALO, became restricted to the POL at mid stage 11 and later localized to Bolwig’s organ precursor cells in the ventral posterior lobe (Fig. 4A-C) (Chang et al., 2001). Fasciclin2 (Fas2) is expressed in the POL of stage 11 embryos where its expression coincides with hh (Fig. 4D,E) (Chang et al., 2001). Fas2 levels in the POL remained robust in hh mutants (Fig. 4F), indicating that Fas2 is not an Hh target and that at least some aspect of the POL remains in the absence of hh function. Full length Ci protein, a hallmark of active Hh signaling, was present in both the POL and adjacent ALO cells at mid stage 11 (Fig. 4G,H), and rdx expression, another hallmark of Hh signaling, had a similar pattern (Fig. 4L). smo was expressed in both lobes, although our in situ hybridization analysis was insufficient to distinguish relative levels of expression within the ALO and POL (Fig. 4I). By contrast, ptc RNA was clearly present only in ALO cells adjacent to the POL (Fig. 4J).

As elevated levels of rdx expression and full length Ci protein are hallmarks of paracrine Hh signaling, their presence in the POL was unexpected. Hh-target genes are not expressed in hh-expressing cells in the wing disc or embryo trunk ectoderm, a pattern that is attributed to repression by En. En also has a role in the positive regulation of hh expression, and loss of rdx expression in the ectodermal stripes of en mutant embryos is one of the consequences (Fig. 4N). However, En is not expressed in either optic lobe (Fig. 4K), and rdx expression remained robust in the optic primordium of en mutant embryos (Fig. 4N). In situ hybridization revealed that rdx RNA was reduced in both the ALO and POL of hh mutants (Fig. 4M), showing that hh function is essential for rdx expression in both regions. The POL is present in hh mutant embryos (Fig. 4F) and expression of rdx in cells dorsal to the POL was not affected by loss of hh (Fig. 4M). In wild type, a two to three cell-wide stripe of rdx expression connected with the dorsal part of the POL (Fig. 4L). This stripe extended dorsally and curved into the anterodorsal part of the head. A similar pattern of eyes absent (eya) expression was also observed (Fig. 5B), and for both genes, this stripe was not altered in hh mutant embryos, consistent with a tissue-specific role for Hh signaling in the POL.

These results indicate that hh expression in the optic primordium is not dependent upon En, and that Hh signal transduction in the optic primordium is activated both in ALO cells (revealed by Ptc, Ci and rdx expression) and in hh-expressing POL cells (revealed by Ci and rdx expression). In the Discussion section, we consider the possibility that hh-expressing cells of the POL respond to Hh in an autocrine manner.

We also monitored visual primordium expression of three genes that are in the set of 2108 with linked DamCi\textsuperscript{Act} and DamCi\textsuperscript{Rep} binding sites: snail (sna), derailed (drl) andeya. They were examined because of their similarly strong upregulation in expression arrays of embryos with ubiquitous Ci\textsuperscript{Act}. sna is one of
the 52 genes in the hh-dependent cluster; expression of the other
two changed in ways that are consistent with regulation by Hh
signaling, albeit at levels that did not meet the clustering thresholds.

In situ hybridization to stage 12 embryos revealed that
sna, drl and eya were expressed in many tissues, and that in the optic
primordium, sna was expressed in both lobes (Fig. 5C) whereas
drl and eya were expressed only in the POL (Fig. 5A,B). Expression
of sna, drl and eya in the optic primordium of hh mutant embryos
was severely reduced or undetectable (Fig. 5D-F). Whereas
expression of sna and eya in other tissues was unaffected in hh
mutants, the ectodermal stripes of drl were absent in mutant
embryos (not shown). With the exception of an increase in the
number of cells that express sna in the peripheral nervous system,
expression of sna, drl and eya in embryos containing ubiquitous
Ci\textsuperscript{Act} was affected only in cells associated with the optic
primordium. In Ci\textsuperscript{Act}-expressing embryos, expression of all three
genes extended from the optic primordium into the dorsal ectoderm
of the head (Fig. 5M-O). These dorsal domains of expression are
similar (or identical) to the Hh-induced cyclopia phenotype in
ptc mutant embryos (which have upregulated Hh signaling) and in
embryos that had been subjected to a heat shock-induced dose of
ectopic Hh expression (Chang et al., 2001).

**Tracheal placode**

The tracheal system arises from twenty clusters that have
approximately 90 epidermal cells each. At stage 11, the tracheal
pits have begun to invaginate, hh is prominently expressed in
posterior compartment epidermal cells to which the pits are
juxtaposed and Hh protein accumulates in the most anterior cells
of the pits (Glazer and Shilo, 2001). A role for Hh signaling in
these anterior pit cells is indicated by their high levels of ptc expression and by the reduced number of cells in the pits of hh mutants (Glazer and Shilo, 2001).

In situ hybridization studies confirmed that hh is expressed in the cells immediately anterior to the pits, and known targets of Hh, such as ci, ptc and rdx were expressed in the adjacent pit cells (Fig. 3; Fig. 6A-D). ptc was expressed most prominently in the anterior portion of the pits; ci was expressed more broadly and most pit cells had elevated levels of full-length Ci protein. Expression array analysis (Fig. 2A) and the DamID assay (Fig. 6Q) led to the identification of Impl2 as an Hh target expressed in the tracheal pit. Impl2 lacks a known function; its expression was activated in anterior cells of the pit (Fig. 6G) and in cells immediately dorsal to the pit invagination (Fig. 6M). It was expressed in only a subset of the cells in the tracheal primordium, which we defined by btl expression (Fig. 6J). Expression of Impl2 was severely reduced but not absent in hh null embryos (Fig. 6H), suggesting that Hh signaling is necessary for activation of Impl2, but that Impl2 also responds to other activators. We monitored Impl2 expression in embryos expressing ectopic CiAct to determine the maximum range of Hh-dependent activation. btlGal4-CiAct embryos had a higher number of Impl2-expressing cells in the tracheal primordium (Fig. 6K), generating an expression pattern in the tracheal primordium that was indistinguishable from btl. Impl2 expression expanded similarly to the entire primordium in daGal4-CiAct. Impl2 expression dorsal to the tracheal primordium was not affected by btlGal4-CiAct (Fig. 6O), suggesting that these cells are not part of the tracheal primordium. Expression in this region is hh-dependent, as indicated by requirement for hh (Fig. 6H) and expansion in daGal4-CiAct (Fig. 6K).

DamCiAct binds two regions at the Impl2 locus; both contain Ci motifs (Fig. 6Q). One of these regions is in the first intron and contains three Ci motifs within a 285 bp sequence; adjacent to this cluster is a consensus Trh binding site (CTCGA). Trh is a basic helix-loop-helix (bHLH)-PAS DNA binding protein that has been implicated in tracheal development (Boube et al., 2000; Wilk et al., 1996). Our evidence suggests that Impl2 is a Trh target. In wild type, expression of trh was immediately dorsal to the tracheal pit invagination (Fig. 6E) and throughout the tracheal primordium. This pattern is similar to btl expression and to Impl2 expression in btlGal4-CiAct embryos. To determine if Trh function is necessary for Impl2 expression, we examined trh mutants. Although Impl2 expression in the cells dorsal to the tracheal primordium was unaffected (Fig. 6N), it was essentially absent in the tracheal primordium (Fig. 6L,N). Based on these observations, we conclude that Trh and Ci together activate Impl2 expression in the tracheal pit.

**DISCUSSION**

Because hh mutant embryos have an extensive syndrome of defects and hh is expressed in most organ systems in the embryo, we designed a global search for genes that are regulated by Hh to improve our understanding of its diverse roles. We used chromatin-binding assays to identify sequences that bind Ci in vivo, analyzed transcription profiles with expression arrays to identify genes whose expression is Hh-dependent, and monitored how several Hh-target genes are expressed and regulated. These experiments illuminate three general properties of Hh signaling: (1) targets of Hh signaling include genes that control cell behavior directly (e.g. cyclins) but a high proportion are transcription factors, with presumed indirect consequences; (2) Hh signal transduction is activated in some Hh-expressing cells where it could be autocrine;
and (3) tissue-specific activation of targets requires the combinatorial activity of both Ci and non-Hh-dependent transcription factors.

**Hh target genes**
Chromatin binding and expression array analyses are, in essence, fractionation methods and, like all such procedures, whether they are genetic screens, biochemical purifications, or behavioral selections, they are plagued with false positives and negatives. Productive use of these methods therefore depends on additional means to extract candidates and determine relevance.

Chromatin-binding assays have a high frequency of false positives, as they detect association with many more sites than are likely to be functionally relevant. Whether binding to non-functional sites is an experimental artifact or represents the actual distribution, it complicates identification of relevant targets. Statistical measures validate the binding assays, but they do not distinguish between the sites. Association with consensus binding sequences might help, but binding to bona fide targets might not always be direct, and our understanding of how accessibility and affinity affect residency is incomplete. It is especially poor for Ci and, although we had hoped that independent identification of binding sites for Ci\textsuperscript{Act} and Ci\textsuperscript{Rep} would establish whether they target the same genes or not, our results were inconclusive. Many of the protected DamID regions are shared and these overlaps are statistically significant; moreover, we show that some of these regions are linked to legitimate targets. However, these chromatin binding experiments cannot rule out the existence of functionally relevant sites that are specific to the activator or repressor forms.

Among the proteins encoded by these 52 targets, we identified six with roles in cell cycle regulation and nineteen that were transcription factors. Previous work has shown that Cyclin D and Cyclin E are Hh targets in the eye disc, that Ci binds to and regulates Cyclin E expression (Duman-Scheel et al., 2002), and that Cyclin E expression in the embryo is regulated in a tissue-specific manner (Jones et al., 2000). Our work adds Cyclin A and Sticky to the cell cycle kinases regulated by Hh. The conceptual significance of these observations is that Hh might promote cell division and growth directly by regulating such cell cycle functions.

In addition, the targets identified by our work and by previous studies show that Hh signaling also regulates cell behavior indirectly. Hh controls growth and patterning of the wing disc by employing Ci to regulate Dpp expression at the anteroposterior compartment border (Tabata et al., 1995; Zecca et al., 1995). Ci also upregulates Wg expression (Lessing and Nusse, 1998), as well as key components of the Notch and EGF signaling pathways (Table 1). Indirect regulation of cell behavior was also revealed by the high proportion of transcription factors among the targets: 19 out of 52 (37%). Transcription factors represent 40% (30 out of 74) of all targets of *Drosophila* Hh identified to date, and the prevalence of transcription factors is consistent with the recent tabulation of Shh targets in the vertebrate limb (Vokes et al., 2007).

**Autocrine Hh signaling**
Hh signaling is understood best in the wing disc and embryo, where posterior compartment cells that express En and Hh export Hh to anterior compartment cells that express neither protein. Although the paracrine response of anterior cells is well-established, posterior cells also activate Hh signaling (Ramirez-Weber et al., 2000) but the basis for their pathway activation has not been settled. Whereas one study concluded that pathway activation is not Hh-dependent (Denef et al., 2000), another concluded that it is (Ramirez-Weber et al., 2000), implying that either Hh-expressing cells respond to Hh received from neighboring cells, or to Hh that they produce themselves.

We do not know if the distinction between autocrine and paracrine signaling is important, but we are intrigued by the responsiveness of Hh-expressing cells to Hh in the optic primordium (Fig. 4). Cells in the POL express hh as well as rdx and have elevated levels of Ci, two hallmarks of pathway activation. POL cells do not express detectable levels of Ptc, an Hh receptor whose negative regulation of the Hh signal transduction pathway is relieved upon Hh binding. Although loss of Ptc function can lead to ectopic pathway activation, expression of rdx in the POL appears not to be a consequence of absent Ptc; rdx expression was markedly reduced in hh mutant embryos, indicating that activation of the pathway in the POL is Hh-dependent. Another possible explanation is that some function other than Ptc keeps the Hh pathway in an ‘off’ state when Hh is not present. Another possibility is that Ptc is expressed in these cells, but at levels too low for our methods to detect, and that, although pathway activation increases ptc expression in other contexts (for instance, in the APL and in every known setting with paracrine signaling), hh-expressing cells of the POL keep ptc expression low. As the elevated level of Ci in the POL does not lead to high levels of ptc, either the Ci is inert or POL cells have a mechanism to override Ci-dependent activation. This mechanism cannot involve En, a negative regulator of ptc, because POL cells do not express en.

In addition to our evidence for autocrine Hh signaling in the wing disc and optic primordium, apparent autocrine signaling by sonic hedgehog has been observed. Three examples are in neural stem cells (Cai et al., 2008), large B-cell lymphoma (Singh et al., 2010) and cerebellar dysplasia in the developing brain (Wang et al., 2004). These examples indicate the potential importance of an autocrine mechanism to human development and disease.

**Tissue-specificity of Hh-dependent responses**
Activation of Hh targets dpp and knot in the wing disc requires participation of Ci with Vestigial (Hepker et al., 1999) or Ataxin-2 Binding Protein 1 (Usa and Shashidhara, 2010), respectively. We show here that activation of Impl2 in the tracheal primordium requires Ci and Trh (Fig. 6). This supports the idea that cell signaling pathways such as Hh are characterized by ‘activator insufficiency’, meaning that pathway activation is necessary, but not sufficient, to induce targets (Barolo and Posakony, 2002).

In the tracheal primordium, our genetic analysis shows that, although trh expression is not dependent on hh, Impl2 expression depends on both hh and trh. These observations indicate that the domain of trh expression must be established independently of Hh and that Impl2 responds only where both Hh signaling and trh expression intersect. As all cells in the tracheal primordium express trh but only a portion express Impl2, the cells in which Hh signaling induces Impl2 expression represent a subset of the cells that are capable of responding. Impl2 is expressed only in cells near Hh-producing cells that, presumably, are exposed to the highest relative levels of Hh. Moreover, as ectopic activation of the pathway led to upregulation of Impl2 expression throughout the tracheal primordium, the level of Hh signaling seems to be adjusted in normal embryos to regulate Impl2. Because Impl2 expression outside of the tracheal primordium is not trh-dependent, we generalize these observations and suggest that the mechanism that leads to selective responsiveness of Hh targets involves both concentration-dependent responses as well as combinatorial control by Hh-sensitive and Hh-independent transcription factors.
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Competing interests statement
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