

# Cadherin Cad99C is regulated by Hedgehog signaling in *Drosophila*

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## Abstract

The subdivision of the *Drosophila* wing imaginal disc into anterior and posterior compartments requires a transcriptional response to Hedgehog signaling. However, the genes regulated by Hedgehog signal transduction that mediate the segregation of anterior and posterior cells have not been identified. Here, we molecularly characterize the previously predicted gene *cad99C* and show that it is regulated by Hedgehog signaling. Cad99C encodes a transmembrane protein with a molecular weight of approximately 184 kDa that contains 11 cadherin repeats in its extracellular domain and a conserved type I PDZ-binding site at its C-terminus. The levels of *cad99C* RNA and protein are low throughout the wing imaginal disc. However, in the pouch region, these levels are elevated in a strip of anterior cells along the A/P boundary where the Hedgehog signal is transduced. Ectopic expression of Hedgehog, or the Hedgehog-regulated transcription factor Cubitus interruptus, induces high-level expression of Cad99C. Conversely, blocking Hedgehog signal transduction by either inactivating Smoothed or Cubitus interruptus reduces high-level Cad99C expression. Finally, by analyzing mutant clones of cells, we show that Cad99C is not essential for cell segregation at the A/P boundary. We conclude that *cad99C* is a novel Hedgehog-regulated gene encoding a member of the cadherin superfamily in *Drosophila*.

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## Introduction

The precise spatial assembly of cells within tissues and organs depends on their ability to recognize, choose, and differentially adhere to neighboring cells (Gumbiner, 1996). A striking example of the ability of cells to recognize and choose neighbors is the formation of non-intermingling sets of cells, termed compartments (Blair, 2003; Dahmann and Basler, 1999; Irvine and Rauskolb, 2001; McNeill, 2000; Tepass et al., 2002; Vincent, 1998). The maintenance of segregated populations of cells at the boundary between adjacent compartments is vital for the partitioning and patterning of invertebrate and vertebrate tissues.

In *Drosophila*, the wing imaginal disc is subdivided into anterior and posterior compartments (Garcia-Bellido et al., 1973). Cells from the two compartments differ in their

abilities to produce and respond to signaling molecules (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Lawrence and Struhl, 1996; Lee et al., 1992; Tabata and Kornberg, 1994; Tabata et al., 1992; Zecca et al., 1995). Cells of the posterior compartment produce, but cannot respond to, the signaling molecule Hedgehog (Hh). In contrast, cells of the anterior compartment do not produce Hh, but can respond to Hh secreted by neighboring posterior cells. As a consequence, Hh unidirectionally signals from posterior to anterior cells. The response to the Hh signal requires the seven-pass transmembrane protein Smoothed (Smo) (Alcedo et al., 1996; van den Heuvel and Ingham, 1996) and the Gli family transcription factor Cubitus interruptus (Ci) (Dominguez et al., 1996). Due to the limited range of Hh movement within the anterior compartment (Chen and Struhl, 1996; Torroja et al., 2004), only an approximately 10-cell wide strip of anterior cells along the anterior–posterior (A/P) compartment boundary transduces the Hh signal. One response to the Hh signal in this strip of cells is production of the long-range signaling molecule

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Decapentaplegic (Dpp), which organizes the growth and patterning of the wing (Hepker et al., 1997; Lecuit et al., 1996; Methot and Basler, 1999; Nellen et al., 1996). Maintained segregation of cells at the A/P boundary is therefore important for positioning and shaping the source of Dpp and thus the patterning of the wing tissue.

The segregation of anterior and posterior cells requires transduction of the Hh signal in anterior cells at the A/P boundary. Mutant anterior cells at the A/P boundary that have lost the ability to respond to Hh no longer segregate with anterior cells, but instead take up positions normally only occupied by posterior cells (Blair and Ralston, 1997; Rodriguez and Basler, 1997). This control of cell segregation by Hh requires the transcription factor Ci, indicating that Hh controls the segregation of anterior and posterior cells by regulating the transcription of target genes (Dahmann and Basler, 2000). However, despite several efforts (e.g., Vegh and Basler, 2003), Hh target genes involved in mediating the segregation of anterior and posterior cells have not been identified.

The current view is that the segregation of cells at compartment boundaries is based on differences in adhesiveness (affinity) of cells in adjacent compartments (Blair, 2003; Dahmann and Basler, 1999; Irvine and Rauskolb, 2001; McNeill, 2000; Tepass et al., 2002; Vincent, 1998). Differential adhesion between cells could be achieved by the presence of either different kinds of cell adhesion molecules or different levels of the same kind of cell adhesion molecule on the cells being segregated (e.g., Friedlander et al., 1989). Cadherins are a class of  $\text{Ca}^{++}$ -dependent cell adhesion molecules that have been implicated in various processes, including the maintenance of epithelial integrity and cell polarity, growth control, synaptic specificity, and morphogenesis (Angst et al., 2001; Tepass et al., 2000; Yagi and Takeichi, 2000). In particular, cadherins can play a role in the segregation of cells. For example, when otherwise non-adhering L cells were transfected with either E- or P-cadherin and then mixed, transfected cells adhered to cells expressing the same subclass of cadherin but segregated away from cells expressing the other subclass of cadherin (Nose et al., 1988). Additionally, in mouse, Cadherin-6 and R-cadherin have been implicated in the segregation of cells at the cortico-striatal compartment boundary (Inoue et al., 2001). Further, DE-cadherin is required for the posterior sorting of the oocyte within the *Drosophila* egg chamber (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998). Interestingly, sorting of the oocyte depends on the relative amount of DE-cadherin, indicating that the sorting of the oocyte is based on the presence of different amounts of a single kind of cadherin in the cells being sorted from one another. Cadherins, especially if expressed differentially in anterior and posterior cells at the A/P boundary, are therefore good candidates for mediating the segregation of anterior and posterior cells.

In *Drosophila*, 17 genes encoding cadherin-like proteins have been predicted (Hill et al., 2001; Hynes and Zhao,

2000). Six of these cadherins, DE-cadherin/shotgun (Oda et al., 1994; Tepass et al., 1996; Uemura et al., 1996), DN-cadherin (Iwai et al., 1997), Flamingo/Starry night (Chae et al., 1999; Usui et al., 1999), Dachshous (Clark et al., 1995), Fat (Mahoney et al., 1991), and Fat-like (Castillejo-Lopez et al., 2004), have been studied in detail; however, none shows a differential expression between anterior and posterior wing imaginal disc cells (Castillejo-Lopez et al., 2004; Clark et al., 1995; Mahoney et al., 1991 and data not shown). Here, we report that the previously predicted, but so far uncharacterized, cadherin Cad99C (Adams et al., 2000; Hill et al., 2001; Hynes and Zhao, 2000) shows a differential expression between anterior and posterior cells at the A/P boundary of wing imaginal discs. Cad99C is expressed at low levels throughout the wing imaginal disc; however, its expression is elevated in a strip of anterior cells along the A/P boundary. We show that the elevated expression of Cad99C is controlled by Ci-mediated Hh signaling. However, clonal analysis using a mutant allele of *cad99C* indicates that Cad99C is not essential to maintain the segregation of anterior and posterior cells.

## Materials and methods

### Molecular cloning

To identify the 5' end of the *cad99C* transcript, total RNA from 100 wing imaginal discs of third instar *y w* larvae was isolated using the RNeasy kit (Qiagen). One fifteenth of the RNA was used as template in a reverse-transcription (RT) PCR (SMART RACE cDNA Amplification Kit, Clontech). The gene-specific primer had the sequence 5'CGGCACACTGGTGGTGTAGCTGTCGTCG3'. The resulting PCR product was cloned using the TOPO TA Cloning kit (Invitrogen) and the inserts of five clones were sequenced. The sequence immediately 5' of the predicted translational start codon (TAAC) matches the *Drosophila* translational start consensus sequence (Cavener, 1987) and is preceded by Stop codons in all three reading frames. A cDNA comprising the entire coding sequence of *cad99C* was obtained by three independent RT-PCR using the primers 5'GGGGTACCTGTTAACGGCGTGTGACTCC3' and 5'TTGCGCGCCAGTTCCTAGTTGTTTCCACC3' (the underlined sequences are *KpnI* and *BssHIII* restriction sites used for cloning), cloned, and sequenced. The predicted amino acid sequence differs at positions 61, 853, 1083, and 1331 from the sequence deposited for Cad99C (accession number AAF56955), presumably due to polymorphisms between the *Drosophila* strains used for sequencing.

### Bioinformatic analysis

The domain organization of Cad99C was predicted using the Simple Modular Architecture Research Tool (SMART),

available at <http://smart.embl-heidelberg.de> (Schultz et al., 1998), with the PFAM domains option activated. The putative extracellular cadherin repeats (ECs) were then retrieved and scanned manually for the presence of Ca<sup>++</sup>-binding sites, according to the consensus sequences DXD, LDRE, and DXNDN (Shapiro et al., 1995). A multiple sequence alignment of the extracellular cadherin repeats (ECs) was obtained with ClustalW v1.4 (included in MacVector 7.2, Accelrys) and color coded to indicate the chemical nature of amino acid residues. The softwares SignalIP (<http://www.cbs.dtu.dk/services/SignalP/>; Nielsen et al., 1997) and Prosite (<http://www.expasy.org/prosite/>; Sigrist et al., 2002) were employed to identify a putative signal peptide sequence at the N-terminus. To analyze the cytoplasmic region for the presence of conserved short motifs, the softwares Prosite and ELM (<http://elm.eu.org/>; Puntervoll et al., 2003) were used, accompanied by manual scanning.

#### Western blot of wing imaginal discs

Third instar larvae were dissected in ice-cold Ringer's solution containing a cocktail of protease inhibitors (Complete-Mini, Roche) and 1 mM PMSF (phenylmethylsulfonyl fluoride). For each sample, 15 wing imaginal discs were collected in ice-cold Ringer's solution containing protease inhibitors. Samples were briefly centrifuged and the pellets were immediately frozen in liquid nitrogen. Pelleted imaginal discs were then boiled in a modified Laemmli sample buffer (2% SDS, 20% glycerol, 125 mM Tris-HCl, pH 6.8) to fully denature the proteins and fractionated under reducing conditions on 10% SDS-polyacrylamide gels. Proteins were then transferred onto a nitrocellulose membrane (Protran, Schleicher and Schuell) (Niessen et al., 1994) and blotted with appropriate primary antibodies (rabbit anti-Cad99C 1:1000 and mouse anti- $\alpha$ -tubulin 1:1000, Sigma T9026) and secondary antibodies (goat anti-rabbit HRP-conjugated 1:5000, Santa Cruz sc-2054 and goat anti-mouse HRP-conjugated 1:5000, Pierce Prod#31430).

#### *Drosophila* stocks

Mutant alleles for *cad99C* were generated by imprecise excision of EP-elements (Rorth, 1996) using standard procedures. The two starting EP-element lines were GE21034 and GE23478 (GenExel, Inc.). Out of 592 excision lines analyzed by PCR, 4 contained deletions in the *cad99C* gene. The allele *cad99C*<sup>57A</sup> contains a genomic deletion of 5382 bp spanning from 4147 bp 5' to 1232 bp 3' of the translational start codon. The coding sequence for the first 101 amino acids is deleted. Homozygous *cad99C*<sup>57A</sup> flies were viable; however, females were sterile. The EP-elements in GE21034 and GE23478 are oriented with the UAS sites facing away from the *cad99C* coding sequence and can therefore not be used to drive expression of *cad99C* in

conjunction with a GAL4 line. To generate an EP-line that can drive *cad99C* expression, we mobilized the EP-elements by crossing the two EP-lines to flies carrying the  $\Delta$ 2–3 transposase. Resulting lines that differed in their eye color from the parental EP-lines were crossed to an *ap-GAL4* driver line (Calleja et al., 1996; active in the dorsal compartment of wing imaginal discs) and the wing imaginal discs of the larval progeny were stained with the Cad99C antibody to detect ectopic Cad99C expression. Two out of 59 lines that were analyzed had elevated levels of Cad99C immunoreactivity throughout the dorsal compartment of the wing imaginal disc. The stronger of these two lines was designated *cad99C*<sup>EP221A</sup>. Additional mutant alleles used were *ci*<sup>94</sup>, a null allele for *ci* (Methot and Basler, 1999), and *smo*<sup>3</sup>, a null allele for *smo* (Alcedo et al., 2000; Chen and Struhl, 1998). The following enhancer trap lines and transgenes were used: *en-lacZ* (Hama et al., 1990), *tub $\alpha$ 1*>CD2, *smo*<sup>+</sup>>*hh* (Dahmann and Basler, 2000), *act5c* > CD2 > GAL4 (Pignoni and Zipursky, 1997), *UAS-ci* (Dahmann and Basler, 2000), *UAS-ci*<sup>PKA4</sup> (Methot and Basler, 2000), and *Pci*<sup>+</sup>*hsp70-GFP* (Methot and Basler, 1999).

#### Clonal analysis

Marked clones of mutant cells were generated by Flp-mediated mitotic recombination (Golic and Lindquist, 1989; Xu and Rubin, 1993), subjecting first instar larvae to a 36–37°C heat-shock for 30 min. Transgenes were expressed using the GAL4-UAS system (Brand and Perrimon, 1993). Genotypes of the larvae were as follows:

*tub $\alpha$ 1* > *hh*: y w *hsp70-flp*; *tub $\alpha$ 1* > CD2,*smo*<sup>+</sup> > *hh*  
*smo*<sup>3</sup>: y w *hsp70-flp*; *smo*<sup>3</sup> FRT40/*ubi-GFP* FRT40  
*ci*<sup>94</sup>: y w *hsp70-flp*; FRT42 *Pci*<sup>+</sup> *hsp70-GFP*/  
 FRT42;;*ci*<sup>94</sup>/*ci*<sup>94</sup>  
*act5c* > GAL4 *UAS-ci*: y w *hsp70-flp*; *UAS-ci*; *act5c* >  
 CD2 > GAL4  
*act5c* > GAL4 *UAS-ci*<sup>PKA4</sup>: y w *hsp70-flp*; *UAS-ci*<sup>PKA4</sup>/  
*act5c* > CD2 > GAL4  
*act5c* > GAL4 *cad99C*<sup>EP221A</sup>: y w *hsp70-flp*; *cad99C*<sup>EP221A</sup>/  
*act5c* > CD2 > GAL4  
*cad99C*<sup>57A</sup>: y w *hsp70-flp*; *en-lacZ*; FRT82 *cad99C*<sup>57A</sup>/  
 FRT82 *hsp70-CD2*  
*cad99C*<sup>57A</sup> Minute: y w *hsp70-flp*; FRT82 *cad99C*<sup>57A</sup>/  
 FRT82 *hsp70-CD2* M(3R)w124

#### Immunohistochemistry

Imaginal discs dissected from late third instar larvae were fixed and stained with appropriate antibodies to mark clones and monitor gene expression. The Cad99C antibody was generated by using a synthetic peptide (YLNDLDRSE-VETTEL) corresponding to amino-acid residues 1691–1706 of Cad99C to immunize rabbits. The resulting serum was affinity purified using the same peptide and used at a 1:10,000 dilution. Additional primary antibodies were

mouse anti-GFP, 1:2000 (Clontech), mouse anti- $\beta$ -Gal, 1:1000 (Promega), mouse anti-CD2, 1:2000 (Serotec), and rabbit anti- $\beta$ -Gal, 1:2000 (Cappel). Secondary antibodies, all diluted 1:200, were goat anti-mouse Alexa 488, goat anti-mouse Alexa 594, goat anti-rabbit Alexa 488, and goat anti-rabbit Alexa 594 (Molecular Probes). Images were recorded on a LSM510 Zeiss confocal microscope.

#### *RNA in situ hybridization*

Larvae were dissected in PBS, transferred to 1.5-ml reaction tubes, and fixed for 20 min in 4% paraformaldehyde in PBS. Dissected larvae were then washed with PBS [treated with 0.1% diethyl pyrocarbonate (DEPC) and then autoclaved], refixed in a solution containing 4% paraformaldehyde, 0.1% Triton X-100, and 0.1% sodium deoxycholate for 20 min, washed twice with PBT (DEPC-treated PBS, 0.1% Tween 20) for 5 min, and then dehydrated through an ethanol series (30%, 50%, 70%, 95%, and 100% ethanol, each 5 min). Dissected larvae were then rinsed once in ethanol:xylene (1:1), once in xylene, incubated in xylene for 3 h, rinsed once in xylene:ethanol (1:1), once in ethanol, 3 times in methanol, once in methanol:PBT (1:1), and once in PBT. Dissected larvae were then washed for 5 min in PBT, fixed in 4% paraformaldehyde in PBS for 20 min, washed twice for 5 min in PBT, and incubated in approximately 0.02 mg/ml proteinase K (Fluka) in PBT (proteinase K concentration was experimentally determined for each batch) for 5 min. Proteinase K digestion was stopped by washing the dissected larvae twice for 1 min with 2 mg/ml glycine. Dissected larvae were then washed twice for 5 min with PBT, fixed in 4% paraformaldehyde for 20 min, and washed 5 times for 5 min in PBT. In preparation for hybridization, dissected larvae were transferred to 1.5-ml safe lock tubes (Eppendorf) containing PBT:hybridization buffer (1:1) [Hybridization buffer (HB) is 50% formamide, 5 $\times$  SSC, 100  $\mu$ g/ml denatured salmon sperm DNA, 50  $\mu$ g/ml heparin, 0.1% Tween 20], and washed for 10 min. Dissected larvae were then washed in HB at 55°C for 10 min and prehybridized in prewarmed HB at 55°C for 1 h. For hybridization, the probe solution (600  $\mu$ l HB containing 6  $\mu$ l of 10 mg/ml denatured salmon sperm DNA and 6  $\mu$ l digoxigenin-labeled riboprobe) was denatured at 80°C for 5 min, added to the dissected larvae, and incubated at 55°C overnight with gentle shaking. Dissected larvae were then washed consecutively in prewarmed HB, HB:PBT (4:1), HB:PBT (3:2), HB:PBT (2:3), HB:PBT (1:4), and twice in PBT for 20 min each at 60°C. For detection, dissected larvae were incubated in anti-digoxigenin antibody coupled to alkaline phosphatase (1:2000 in PBT; Roche) for 90 min at room temperature, washed 4 times in PBT for 20 min each, washed twice in freshly prepared buffer B3 (100 mM NaCl, 50 mM MgCl<sub>2</sub>, 100 mM Tris HCl, pH 9.5, 0.1% Tween 20) for 5 min each, and then incubated in staining solution [buffer B3 containing 0.675 mg/ml nitro-tetrazolium blue chloride (Fluka) and 0.35 mg/ml 5-Bromo-4-chloro-3-indolylphosphate *p*-toluidine (Fluka)] in the dark.

When appropriate staining was obtained, the color reaction was stopped by washing twice in PBT. After dehydration (as described above, except 30 s each), imaginal discs were mounted on glass slides in Canada Balsam (Fluka) dissolved in methylsalicylate (Fluka). Digoxigenin-labeled riboprobes were made using the DIG RNA Labeling Kit (10  $\mu$ l reaction volume; Roche). Following *in vitro* transcription, 10  $\mu$ l DNase I solution [10 U DNase I (RNase-free; Roche) 1 $\times$  DNaseI buffer, 10 mM DTT] was added to the reaction and incubated for 15 min at 37°C. The probe was then partially hydrolyzed by adding 80  $\mu$ l hydrolysis buffer (125 mM NaCO<sub>3</sub>, pH 10.2) and incubating it for 20 min at 60°C. The probe was then precipitated by adding 50  $\mu$ l 7.5 M ammonium acetate and 400  $\mu$ l ethanol followed by centrifugation. The precipitated probe was resuspended in 50  $\mu$ l resuspension buffer (50% formamide, 0.5 $\times$  TE, pH 7.4, 0.1% Tween 20). For generating the *cad99C* antisense RNA probe, plasmid LD23052 (containing the 3' most 2.5 kb cDNA of *cad99C*; BDGP) was linearized with *EcoRV* followed by *in vitro* transcription using SP6 RNA polymerase.

## Results

### *cad99C* encodes a cadherin

To identify genes mediating the segregation of cells at the A/P boundary, we determined the expression pattern of genes predicted to encode cell adhesion molecules in wing imaginal discs from third instar larvae by RNA *in situ* hybridization. We then selected genes that were expressed at different levels in anterior and posterior cells for further study. Here, we report the characterization of one of the genes identified, the predicted gene *cad99C*. We first determined its genomic organization. The 3' region of the *cad99C* transcript was represented by an Expressed Sequence Tag (EST) that was available from the Berkeley Drosophila Genome Project (BDGP). To identify the 5' region of the transcript, we used Rapid Amplification of cDNA Ends (RACE). RACE identified two distinct transcripts that differed in their 5' untranslated regions (Fig. 1A). Conceptual translation of the coding sequence revealed a protein of 1706 amino acids with a predicted molecular weight of 184 kDa. Sequence analysis identified a signal peptide at its N-terminus followed by 11 extracellular cadherin (EC) domains and a single putative transmembrane domain comprising amino acids 1397–1419 (Fig. 1B). Thus, Cad99C appears to be a member of the cadherin superfamily. Cadherin domains are stabilized by the binding of Ca<sup>++</sup> ions between adjacent cadherin domains and each of the 11 EC domains in Cad99C contains at least one consensus sequence for mediating Ca<sup>++</sup> binding (Fig. 2). The intracellular domain of Cad99C is predicted to contain the following sequence motifs that are conserved with a putative Cad99C homolog from *Anopheles gambiae* (accession number XP\_312660): two low-complexity (LC) regions, one consensus phosphor-

ylation site for protein kinase C (position 1483–1485), two consensus phosphorylation sites for casein kinase II (positions 1697–1701 and 1702–1706), and at its C-terminus a class I PDZ (PSD-95, Dlg, ZO-1) binding site (Nourry et al., 2003). A  $\beta$ -catenin binding site, as identified in type-I and type-II cadherins and DE-cadherin (Nollet et al., 2000; Oda et al., 1994), was not found, indicating that Cad99C does not belong to these subfamilies of cadherins.

We conclude that Cad99C is a new member of the cadherin superfamily in *Drosophila*.

*Cad99C* is expressed at high levels in a strip of cells along the A/P boundary in wing imaginal discs

The expression pattern of *cad99C* in wing imaginal discs was determined by generating a *cad99C*-specific RNA probe and performing RNA in situ hybridization on wing imaginal discs of third instar larvae. A weak hybridization signal was observed throughout the wing imaginal disc (Fig. 4A). However, the strength of the hybridization signal was increased in a strip of cells in the middle of the wing imaginal disc pouch parallel to the A/P boundary, a first indication that Cad99C expression might be regulated by Hh signaling. To more precisely map the domain of high-level Cad99C expression and to test whether Cad99C expression is regulated by Hh signaling, we generated an antibody against Cad99C (see Materials and methods). To test the specificity of the anti-Cad99C antibody, we performed Western blot analysis using extracts from wing imaginal discs from larvae heterozygous mutant for *cad99C*, homozygous mutant for *cad99C*, or overexpressing Cad99C using an *act5c-GAL4* driver in conjunction with the *cad99C*<sup>EP221A</sup> EP (enhancer/promoter; Rorth, 1996) line. A mutant allele of *cad99C*, termed *cad99C*<sup>57A</sup>, was generated by imprecise excision of an EP-element located in the first intron of *cad99C* (see Materials and methods). As shown in Fig. 3, a protein of approximately 184 kDa, the predicted size of Cad99C, was identified by our antibody in extracts from heterozygous mutants but was absent in extracts from homozygous mutants. In addition, there was an increased abundance in the 184-kDa band in extracts from wing imaginal discs overexpressing Cad99C. Thus, the anti-Cad99C antibody detects Cad99C protein.

As our antibody recognized Cad99C protein, we next used the anti-Cad99C antibody to stain wing imaginal discs in order to determine the distribution of the Cad99C protein in this tissue. Weak immunoreactivity was detected throughout the wing imaginal disc with increased immunoreactivity present in a strip of cells about 10-cell-diameter in width in the center of the wing imaginal disc pouch (Fig. 4B). The pattern of Cad99C immunoreactivity resembled the distri-

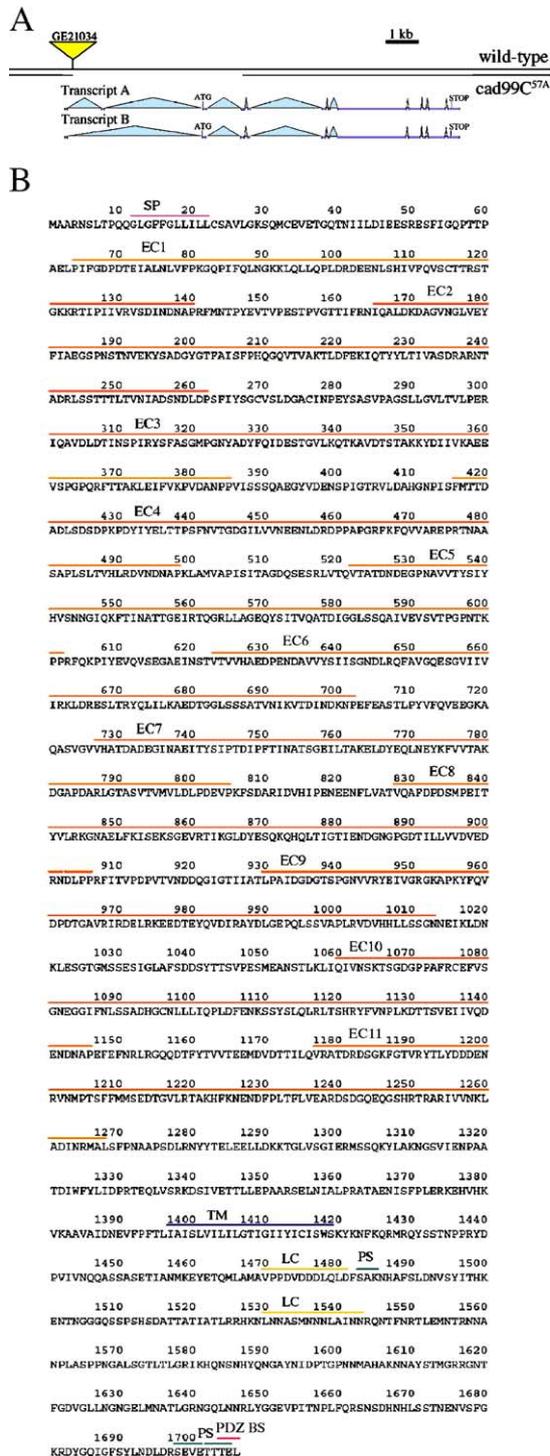


Fig. 1. Bioinformatic analysis of Cad99C protein and gene organization. (A) Organization of the *cad99C* gene and extent of the deletion in the *cad99C*<sup>57A</sup> allele. Two different transcripts for *cad99C* have been detected (Transcript A and B), differing in the presence of an exon in the 5'-untranslated region. The position of the EP-element GE21034, which was used to generate the allele *cad99C*<sup>57A</sup>, is indicated. (B) Amino acid sequence and domain structure of cadherin Cad99C. The extracellular region contains a signal peptide sequence (SP, pink) and 11 extracellular cadherin repeats (EC1 to EC11, orange). A transmembrane region (TM, blue) separates the extracellular from the intracellular region, which contains low-complexity regions (LC, yellow), putative phosphorylation sites for protein kinase C and casein kinase II (PS, green), and a C-terminal type I PDZ-binding site (PDZ BS, red).

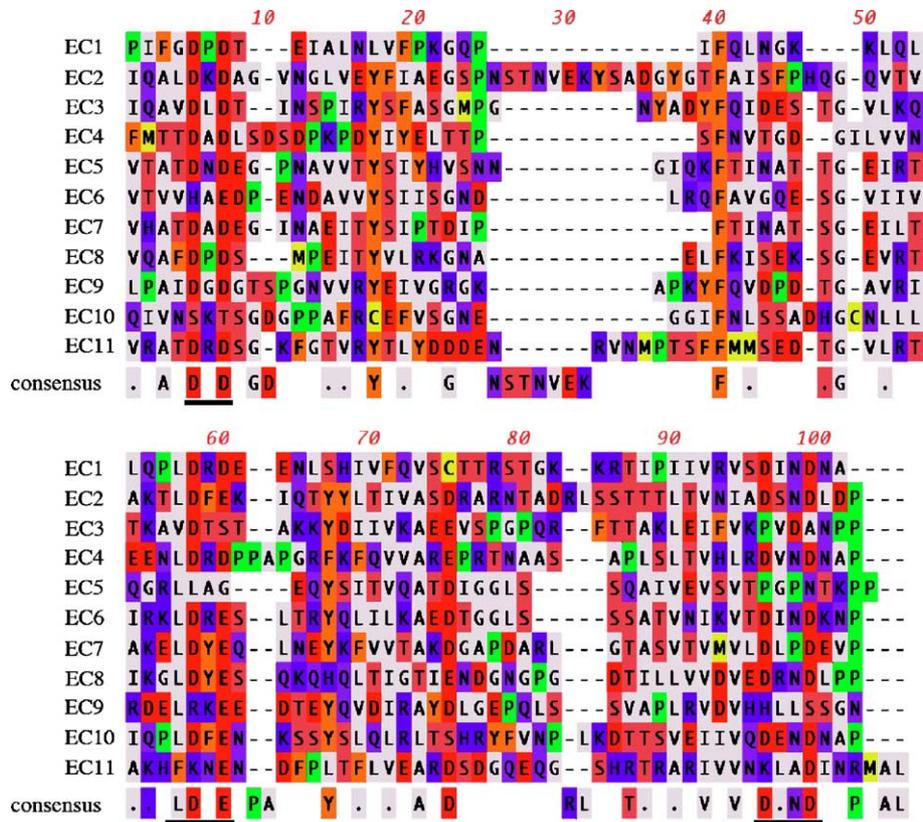


Fig. 2. Multiple sequence alignment of Cad99C extracellular cadherin repeats (ECs). Amino acid residues are color coded on the basis of their chemical nature: acidic (DE): red; hydrophobic (AGILV): grey; amido (NQ): light blue; aromatic (FWY): orange; basic (RHK): dark blue; hydroxyl (ST): pink; proline (P): green; sulfur (CM): yellow. Consensus Ca<sup>++</sup>-binding sites are underlined (in black).

bution of *cad99C* RNA (Fig. 4A), suggesting that the expression of Cad99C is regulated at a transcriptional level. Immunoreactivity was mainly detected at what appeared to be cell boundaries, indicating that Cad99C resides in the plasma membrane of wing imaginal disc cells. To test the specificity of the anti-Cad99C antibody in immunofluorescence preparations, we stained wing imaginal discs containing clones of cells homozygous mutant for *cad99C*<sup>57A</sup> with the antibody. As shown in Fig. 5A, Cad99C immunoreactivity was highly reduced in homozygous *cad99C*<sup>57A</sup> clones, indicating that the immunoreactivity is specific for Cad99C protein.

To precisely determine the localization of the strip of cells expressing Cad99C at high levels relative to the A/P boundary, we used the *en-lacZ* enhancer trap line to mark the posterior compartment and stained wing imaginal discs for Cad99C. The strip of cells showing high Cad99C immunoreactivity was precisely abutting, but not overlapping, the *en-lacZ* staining, indicating that high-level Cad99C expression is in a strip of anterior cells directly next to the A/P boundary (Fig. 4C).

#### *Hh* can induce high-level expression of Cad99C

Results from the above experiments showed that the expression of Cad99C is elevated in a strip of anterior cells

along the A/P boundary of wing imaginal discs, cells known to respond to the Hh signal. To test whether Hh could increase Cad99C expression, we expressed *hh* from a transgene in clones of cells and monitored Cad99C levels by immunofluorescence. In a wild-type wing imaginal disc, the Hh signal can only be transduced in the anterior, but not the posterior, compartment (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Consistent with this Cad99C immunoreactivity was increased in clones of cells expressing Hh located in the anterior, but not posterior compartment (Fig. 4D). Increased Cad99C immunoreactivity was not only observed within anterior clones expressing Hh but also in a few rows of cells outside of the clone, consistent with the ability of Hh to spread within the tissue. We conclude that Hh can induce high-level expression of Cad99C.

#### *Smo*-dependent Hh signal transduction is required for high-level Cad99C expression

To test whether Hh signal transduction is required for high-level Cad99C expression, we monitored the expression of Cad99C in clones of cells lacking the ability to transduce the Hh signal due to a mutation in *smoothened* (*smo*), a gene encoding a transmembrane protein essential for Hh signal transduction (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Cad99C immunoreactivity was reduced to

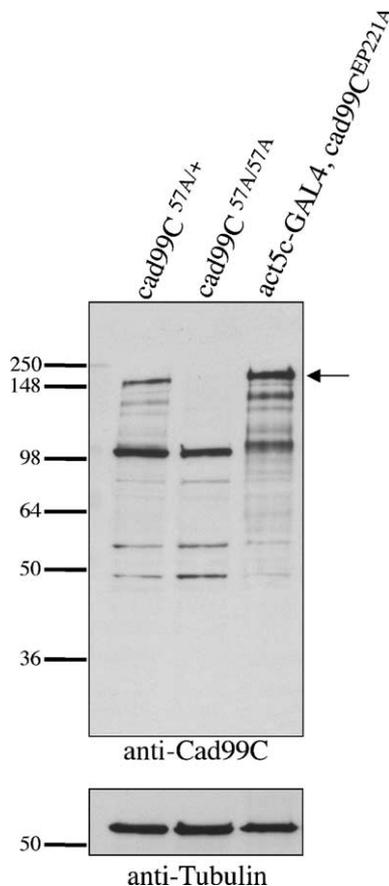


Fig. 3. Western blot analysis of wing imaginal discs with an affinity-purified anti-Cad99C antibody. Wing imaginal discs were collected from heterozygous *cad99C*<sup>57A/+</sup> (lane 1) and homozygous mutant *cad99C*<sup>57A/57A</sup> larvae (lane 2), and from larvae overexpressing Cad99C (*act5c-GAL4, cad99C*<sup>EP221A</sup>; lane 3). An anti-Cad99C antibody detects a protein of ~184 kDa (arrow), the expected molecular weight for Cad99C, in heterozygous *cad99C*<sup>57A/+</sup> (lane 1) and Cad99C overexpression (lane 3) extracts. No immunoreactivity against this ~184-kDa protein is observed in *cad99C*<sup>57A/57A</sup> mutant wing imaginal disc extracts (lane 2). Immunoreactivity against the ~184-kDa protein is increased when Cad99C is overexpressed (lane 3). In addition to the ~184-kDa protein, a few other proteins are recognized by the Cad99C antiserum. However, these proteins are still detected in extracts from mutant *cad99C*<sup>57A/57A</sup> wing imaginal discs. Subsequent blotting with an anti  $\alpha$ -tubulin antibody (lower panel) shows that equal amounts of protein were loaded in each lane. Molecular weights are indicated to the left (in kDa).

the low level observed in cells far away from the A/P boundary in *smo* mutant clones located in anterior cells close to the A/P boundary (Fig. 4E). This indicates that Smo-mediated Hh signal transduction is required for high-level Cad99C expression in a strip of anterior cells along the A/P boundary.

#### High-level Cad99C expression requires Ci

Hh signal transduction also requires the transcription factor Ci (Dominguez et al., 1996). Ci is only expressed in anterior cells, where it is posttranscriptionally regulated (Aza-Blanc et al., 1997; Eaton and Kornberg, 1990; Methot and Basler, 1999; Ohlmeyer and Kalderon, 1998; Price and

Kalderon, 1999). In anterior cells far away from the A/P boundary that receive little or no Hh, the 155-kDa full-length form of Ci is proteolytically cleaved generating a 75-kDa fragment that acts as a repressor of transcription (in the following referred to as Ci[rep]). In cells receiving and transducing the Hh signal, proteolysis is prevented and the full-length form is converted into an activator of transcription (in the following referred to as Ci[act]). To test whether expression of Cad99C requires Ci, we monitored the expression of Cad99C in *ci* mutant clones. Cad99C immunoreactivity was reduced to the low level observed in cells far away from the A/P boundary in *ci* mutant clones located in the anterior compartment close to the A/P boundary (Fig. 4F), indicating that Ci is required for high-level Cad99C expression. Cad99C immunoreactivity was not detectably changed in *ci* mutant clones located in the anterior compartment away from the A/P boundary or within the posterior compartment (Fig. 4F), indicating that the low-level Cad99C expression does not depend on Ci. Further, these results also suggest that in wild-type anterior cells far away from the A/P boundary, Ci[rep] is not required to repress *cad99C*.

#### Ci[act] induces high-level Cad99C expression

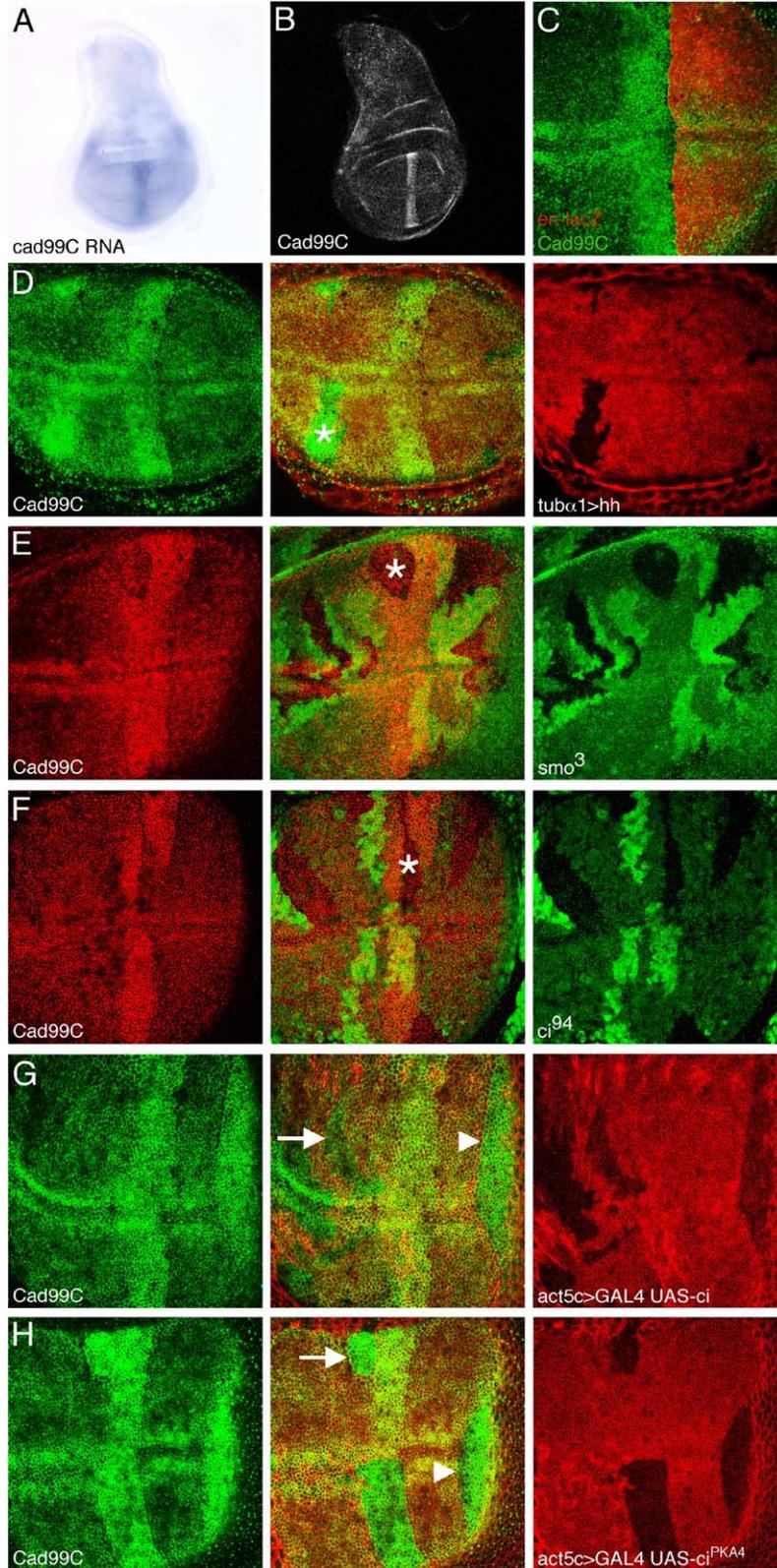
As the above results indicated that Ci is necessary for high-level Cad99C expression, we next addressed whether Ci is also sufficient to induce high-level expression of Cad99C. To this end, we analyzed Cad99C protein level in clones of cells expressing Ci from a transgene using the GAL4-UAS system (Brand and Perrimon, 1993). In posterior cells, under the influence of Hh, Ci is converted to Ci[act]. In contrast, in anterior cells far away from the boundary, Ci is proteolytically cleaved to Ci[rep]. Ci-expressing clones located in the posterior compartment of the wing imaginal disc pouch, but not in the anterior compartment, showed increased immunoreactivity for Cad99C (Fig. 4G). This suggests, first, that Ci is sufficient to induce high-level Cad99C expression in the pouch region of the wing imaginal disc and, second, that it is the Ci[act] form of Ci that induces high-level expression of Cad99C. Clones of cells expressing a constitutively active form of Ci which cannot be proteolytically cleaved to Ci[rep], Ci<sup>PKA4</sup> (Methot and Basler, 2000), induced high-level Cad99C immunoreactivity in both the anterior and posterior compartments (Fig. 4H). Taken together, these results indicate that Ci[act]-mediated Hh signaling is necessary and sufficient for high-level Cad99C expression in a strip of cells along the A/P boundary of the wing imaginal disc pouch.

#### Cad99C is not required to maintain the segregation of anterior and posterior cells

We have shown above that Ci[act]-mediated Hh signaling induces high-level expression of Cad99C in a strip of anterior cells along the A/P boundary. Previously, Ci[act]-

mediated Hh signal transduction has been shown to be required in anterior cells at the A/P boundary for them to segregate from posterior cells (Dahmann and Basler, 2000). Thus, this raises the possibility that Cad99C might be one of the genes regulated by Hh signaling that is involved in

maintaining the segregation of anterior and posterior cells. To test this possibility, we generated clones of cells homozygous mutant for *cad99C*<sup>57A</sup> and analyzed their segregation at the A/P compartment boundary. The location of the A/P boundary was visualized using the posterior-



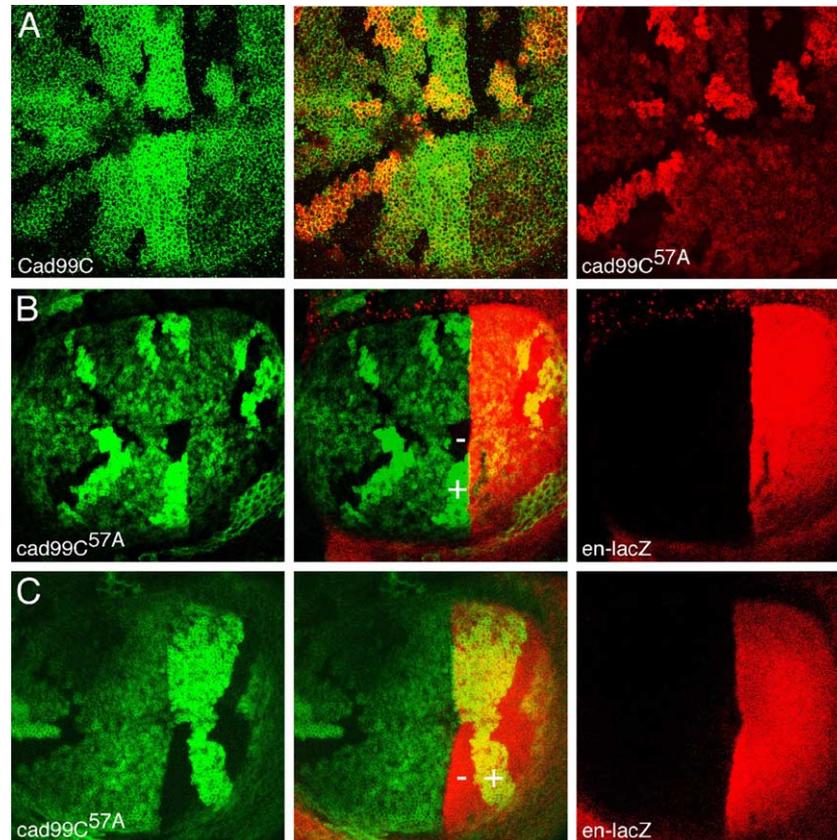


Fig. 5. Cad99C is not essential for the segregation of anterior and posterior cells. (A) Clones of cells homozygous mutant for *cad99C*<sup>57A</sup> are marked by the absence of CD2 staining (red). Cad99C staining (green) is highly reduced in *cad99C*<sup>57A</sup> clones. (B, C) Clones of cells homozygous mutant for *cad99C*<sup>57A</sup> are marked by the absence (–) of CD2 staining (green). Sibling clones are marked by elevated CD2 staining (+). The posterior compartment is marked by the activity of the *en-lacZ* enhancer trap line (red). (B) *cad99C*<sup>57A</sup> clones of anterior origin (as judged by the position of the sibling clone and the absence of *en-lacZ* expression) remain in the anterior compartment. (C) *cad99C*<sup>57A</sup> clones of posterior origin (as judged by the position of the sibling clone and *en-lacZ* expression) remain in the posterior compartment.

specific *en-lacZ* enhancer trap line (Hama et al., 1990). We analyzed 32 *cad99C*<sup>57A</sup> homozygous mutant clones of anterior and 37 *cad99C*<sup>57A</sup> mutant clones of posterior origin located at the A/P boundary. All mutant clones remained entirely in the compartment in which they had been generated (Figs. 5B,C). A similar result was obtained when homozygous mutant *cad99C*<sup>57A</sup> clones were generated in heterozygous *Minute*<sup>+</sup> animals (Morata and Ripoll, 1975), thereby giving the *cad99C*<sup>57A</sup> cells a growth advantage (data not shown). Taken together, these results indicate that Cad99C is not required to maintain the segregation of anterior and posterior cells at the A/P boundary. Further, posterior cells overexpressing Cad99C (*act5c > GAL4, cad99C*<sup>EP221A</sup>) segregated normally from anterior cells

(data not shown), indicating that high-level Cad99C expression is not sufficient to confer anterior-type segregation properties to posterior cells.

## Discussion

Members of the Hh family of secreted signaling molecules play important roles during the development of invertebrates and vertebrates and inappropriate Hh signaling activity is associated with numerous diseases in humans (reviewed by McMahon et al., 2003). The Hh signaling pathway leads to the activation of transcription factors of the Ci/Gli family (reviewed by Bijlsma et al., 2004; Kalderon,

Fig. 4. Cad99C expression is regulated by Hh signaling. (A) A wing imaginal disc hybridized with a *cad99C*-specific RNA probe. (B) A wing imaginal disc stained with the anti-Cad99C antibody. (C) Wing imaginal disc stained for the posterior-specific activity of the *en-lacZ* enhancer trap line (red) and Cad99C (green). Elevated levels of Cad99C are detected in a strip of anterior cells along the A/P boundary. (D) Clones of cells expressing Hh (*tubx1 > hh*) are marked by the absence of CD2 staining (red). Cad99C staining (green) is elevated in anterior clones (asterisk) and surrounding cells. (E) Clones of cells homozygous mutant for *smo*<sup>3</sup> are marked by the absence of GFP staining (green). Cad99C staining (red) is reduced in anterior *smo*<sup>3</sup> clones located close to the A/P boundary (asterisk). (F) Clones of cells homozygous mutant for *ci*<sup>94</sup> are marked by the absence of GFP staining (green). Cad99C staining (red) is reduced in anterior *ci*<sup>94</sup> clones located close to the A/P boundary (asterisk). (G) Clones of cells expressing Ci (*act5c > GAL4, UAS-ci*) are marked by the absence of CD2 staining (red). Posterior (arrowhead), but not anterior (arrow), clones show elevated Cad99C staining (green). (H) Clones of cells expressing a constitutively active form of Ci (*act5c > GAL4, UAS-ci*<sup>PKA4</sup>) are marked by the absence of CD2 staining (red). Both anterior (arrow) and posterior (arrowhead) clones show elevated Cad99C staining (green). In this figure and in Fig. 5, wing imaginal discs from third instar larvae are shown with the anterior to the left and dorsal up.

2004). To understand how Hh controls developmental processes, it is therefore important to identify genes regulated by Hh. However, given the many processes in which Hh signaling is involved, only comparatively few genes regulated by Hh have been described. Here, we have identified a gene in *Drosophila*, *cad99C*, which is regulated by Ci-mediated Hh signaling and encodes a member of the cadherin superfamily.

#### *cad99C* encodes a member of the cadherin superfamily

The predicted Cad99C amino acid sequence contains the characteristic features common to members of the cadherin superfamily including a signal peptide, cadherin domains flanked by Ca<sup>++</sup>-binding sites, and a transmembrane domain, defining Cad99C as a member of this superfamily. A  $\beta$ -catenin binding site, which is present in the cytoplasmic domain of type-I and type-II cadherins providing a link to the actin cytoskeleton (Angst et al., 2001; Nollet et al., 2000), was not identified. However, we did identify a conserved type I PDZ-binding sequence at the C-terminus of Cad99C. PDZ domain-containing proteins commonly act as linkers that facilitate the assembly of large molecular complexes within cells (Nourry et al., 2003), suggesting that Cad99C physically interacts with a cytosolic protein complex. Only few other cadherins have been reported to contain C-terminal PDZ-binding sequences (Boeda et al., 2002; Moulton et al., 2004; Okazaki et al., 2002; Siemens et al., 2002). Cadherin 23, for example, a vertebrate cadherin lacking the consensus R1 and R2  $\beta$ -catenin binding sites, associates with its C-terminal PDZ-binding site with the PDZ domain-containing protein harmonin b (Boeda et al., 2002; Siemens et al., 2002). Interestingly, harmonin b has been shown to be able to bundle actin filaments, providing a potential link between cadherin 23 and the actin cytoskeleton independent of  $\beta$ -catenin (Boeda et al., 2002). It is therefore conceivable that Cad99C interacts with a PDZ domain-containing protein that provides a link to the actin cytoskeleton independent of  $\beta$ -catenin. The PDZ-binding sequence of Cad99C is adjacent to and overlapping with two predicted Ser/Thr kinase phosphorylation sites. The interaction between a PDZ-binding peptide and a PDZ domain can be disrupted by phosphorylation of the PDZ-binding sequence by Ser/Thr kinases (Nourry et al., 2003), suggesting that the potential binding of Cad99C to a PDZ domain-containing protein could be regulated by phosphorylation.

#### *Hedgehog* regulates *cad99C* transcriptional activity

Cad99C is expressed at low levels throughout the wing, haltere, and leg imaginal discs (Figs. 4A,B and data not shown), whereas elevated levels of Cad99C expression are confined to a strip of cells along the A/P boundary of the wing imaginal disc pouch that is known to respond to the Hh signal. Even though anterior cells along the A/P boundary of haltere and leg imaginal discs as well as cells

outside the pouch region of wing imaginal discs also respond to the Hh signal, no elevated level of Cad99C was observed in these cells, indicating that *cad99C* is a region-specific Hh target gene. The Cad99C protein profile resembles *cad99C* RNA levels, indicating that the elevated expression of Cad99C is mainly due to transcriptional and not translational or posttranslational regulation. High-level Cad99C expression was reduced to the low level present in cells far away from the A/P boundary in clones of cells lacking Hh signal transduction due to mutations in either *smo* or *ci*. Conversely, ectopic expression of either Hh or Ci was sufficient to increase Cad99C expression in the wing imaginal disc pouch, indicating that high-level *cad99C* expression is controlled by Ci-mediated Hh signaling.

Different Hh-regulated genes respond differently to Ci[act] and Ci[rep]. For example, the expression of *dpp* is regulated both by Ci[act] and Ci[rep], whereas *hh* and *ptc* only respond to one form of Ci, Ci[rep] or Ci[act], respectively (Methot and Basler, 1999). Like *ptc*, *cad99c* appears to respond exclusively to Ci[act]. We infer this from five observations. First, ectopic expression in posterior cells of Ci, which under the influence of Hh is converted to Ci[act], induced high-level *cad99C* expression. Second, misexpression of a constitutively active form of Ci, Ci<sup>PKA4</sup> (Methot and Basler, 2000), also induced high levels of *cad99C* expression. Third, *ci* null mutant clones in the anterior compartment close to the A/P boundary, where Ci[act] is the predominant form of Ci, failed to upregulate *cad99C* expression. Fourth, expression of a constitutive repressor form of Ci, Ci<sup>Cell</sup> (Methot and Basler, 1999), did not reduce the low-level expression of *cad99C* (data not shown). Fifth, *ci* null mutant clones in the anterior compartment away from the A/P boundary, where Ci[rep] is the prevailing form of Ci, showed no increase in the expression of *cad99C*. Taken together, we conclude that *cad99C* expression is regulated by Ci[act] and not Ci[rep].

#### *Cad99C* is not essential for the segregation of anterior and posterior cells

The segregation of cells at compartment boundaries is thought to depend on the differential adhesiveness (affinity) of cells on both sides of the compartment boundaries. Based on thermodynamic considerations, Steinberg has proposed that cells will maximize the total strength of their adhesive interactions with neighboring cells by replacing weak cell–cell interactions with stronger ones (Steinberg, 1963). Cells with strong adhesive interactions will thus associate preferentially with one another and will segregate from less avidly adhering cells. As predicted by this model, cells expressing different levels of the same adhesion molecule segregate from one another (Friedlander et al., 1989; Steinberg and Takeichi, 1994). However, few adhesion molecules have been identified that can promote the differential adhesiveness of cells at compartment boundaries (e.g., Milan et al., 2001).

The maintenance of the A/P boundary in the developing *Drosophila* wing requires Ci-mediated Hh signal transduction in anterior cells (Dahmann and Basler, 2000). This suggests that Hh signaling may regulate the transcription of one or more genes that in turn affect the adhesiveness of anterior cells. Members of the cadherin superfamily are known to mediate adhesion between cells and several cadherins have been shown to be involved in cell segregation (see Introduction). Even though most cadherins implicated so far in cell segregation contain cytoplasmic  $\beta$ -catenin binding sites, which are absent in Cad99C, several cadherins lacking  $\beta$ -catenin binding sites have also been shown to mediate cell segregation (e.g., Kuroda et al., 2002). The discovery of a gene that is both regulated by Hh signaling and encodes for a cadherin, therefore, provides an attractive candidate for mediating the segregation of anterior and posterior cells. As outlined above, *cad99C* expression is not elevated in cells along the A/P boundary of haltere and leg imaginal discs or outside the pouch region of wing imaginal discs, indicating that if the elevated expression of Cad99C were important for cell segregation, this could not be a general mechanism for segregating anterior and posterior cells. However, as wing imaginal disc pouch cells differ in their expression profile from wing imaginal disc cells outside of the pouch (Mann and Morata, 2000), it is not inconceivable that different molecules could operate to segregate cells at the A/P boundary in different regions of the wing imaginal disc or in different imaginal discs.

In this study, we generated a mutant allele of *cad99C*, termed *cad99C<sup>57A</sup>*, in order to test whether Cad99C is required to segregate anterior and posterior cells. *cad99C<sup>57A</sup>* appears to be a null allele of *cad99C* based on four criteria. First, sequencing of the genomic DNA revealed that the predicted promoter region, the transcriptional start site, and the coding sequence for the first 101 amino acid residues were deleted. Second, an RNA probe recognizing the 3' region of the *cad99C* transcript, outside of the deletion present in *cad99C<sup>57A</sup>*, did not show detectable staining in wing imaginal discs from homozygous *cad99C<sup>57A</sup>* mutant larvae (data not shown), indicating that the *cad99C* transcript levels were highly reduced. Third, an antibody directed to the C-terminus of Cad99C did not recognize a protein of the predicted size for Cad99C in extracts from wing imaginal discs of homozygous *cad99C<sup>57A</sup>* mutant larvae. Fourth, Cad99C immunoreactivity was highly reduced in homozygous *cad99C<sup>57A</sup>* mutant clones within wing imaginal discs.

The requirement for Cad99C to maintain the segregation of anterior and posterior wing imaginal disc cells was tested by clonal analysis. Using this assay, Cad99C was found not to be essential for maintaining the normal segregation of anterior and posterior cells. This result can be explained in several ways. First, Cad99C does not play any role in the segregation of anterior and posterior cells. The Hh-dependent increase in expression of Cad99C may either be irrelevant for the function of Cad99C or might reflect an

unrelated function. For example, Hh signaling is required for the patterning of the longitudinal wing veins L3 and L4 (De Celis, 2003). It is thus conceivable that Cad99C may play a role in this aspect of Hh signaling. However, vein patterning appeared to be normal in wings from homozygous *cad99C<sup>57A</sup>* mutants (data not shown). Second, the activity of Cad99C in mediating the segregation of anterior and posterior cells is redundant with the activity of one or several of the remaining 16 cadherins in *Drosophila*. Third, different, partially redundant mechanisms contribute to the segregation of anterior and posterior cells. For example, one mechanism might be Cad99C-dependent whereas additional mechanisms may rely on cell surface proteins unrelated to cadherins or on cytoskeletal components.

The identification of *cad99C* as an Hh-regulated gene provides a starting point to investigate a cell biological mechanism used by Hh signaling to control the development of the *Drosophila* wing. It also provides a further step towards the functional characterization of all remaining members of the cadherin superfamily present in *Drosophila* that have so far only been predicted based on the genomic sequence.

The GenBank accession numbers for transcripts A and B of *cad99C* are AY853686 and AY853687, respectively.

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