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Hedgehog and Dpp signaling induce cadherin Cad86C expression in the morphogenetic furrow during *Drosophila* eye development

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ABSTRACT

During *Drosophila* eye development, cell differentiation is preceded by the formation of a morphogenetic furrow, which progresses across the epithelium from posterior to anterior. Cells within the morphogenetic furrow are apically constricted and shortened along their apical–basal axis. However, how these cell shape changes and, thus, the progression of the morphogenetic furrow are controlled is not well understood. Here we show that cells simultaneously lacking Hedgehog and Dpp signal transduction fail to shorten and do not enter the morphogenetic furrow. Moreover, we have identified a gene, cadherin *Cad86C*, which is highly expressed in cells of the leading flank of the morphogenetic furrow. Ectopic activation of either the Hedgehog or Dpp signal transduction pathway results in elevated Cad86C expression. Conversely, simultaneous loss of both Hedgehog and Dpp signal transduction leads to decreased Cad86C expression. Finally, ectopic expression of Cad86C in either eye-antennal imaginal discs or wing imaginal discs results in apical constriction and shortening of cells. We conclude that Hedgehog and Dpp signaling promote the shortening of cells within the morphogenetic furrow. Induction of Cad86C expression might be one mechanism through which Hedgehog and Dpp promote these cell shape changes.

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1. Introduction

Changes in the shape of cells are important for the morphogenesis of developing animals. Animal development is, to a large extent, driven by the activities of signaling molecules and their downstream transduction pathways. The shape of individual cells depends mainly on the cytoskeleton and its associated proteins. How signal transduction pathways impinge on the activities of molecules associated with the cytoskeleton in order to bring about cell shape changes is poorly understood.

The eye imaginal disc of *Drosophila melanogaster* is a model in which to study developmentally regulated cell shape

changes. The eye imaginal disc is a sac-like structure composed of a single-layered epithelium (Fig. 1A and B). Cells on one side of the eye imaginal disc are squamous and cells on the other side are highly columnar (Ready et al., 1976). During eye imaginal disc development, rows of columnar cells transiently change their shape. Cells shorten along their apicobasal axis and constrict apically resulting in the formation of an epithelial fold known as the morphogenetic furrow (Ready et al., 1976; Tomlinson, 1985). In contrast to most other epithelial folds, the morphogenetic furrow does not remain in a particular position; instead, it moves across the epithelium from the posterior margin of the eye imaginal disc towards the anterior. Progression of the morphogenetic furrow is asso-

ciated with cell differentiation. Shortly after emerging from the morphogenetic furrow, cells arrange into clusters and differentiate into photoreceptors (reviewed by Baker, 2007; Hsiung and Moses, 2002).

The wave of photoreceptor differentiation, which follows the morphogenetic furrow, is driven by activity of the Hedgehog signaling molecule. Hedgehog is expressed in the differentiating photoreceptor cells posterior to the morphogenetic furrow (Heberlein et al., 1993; Ma et al., 1993). Lack of Hedgehog results in severe retardation or halt of the photoreceptor differentiation front (Heberlein et al., 1993; Ma et al., 1993). Moreover, photoreceptor differentiation is delayed in clones of cells mutant for smoothened (smo) (Greenwood and Struhl, 1999; Strutt and Mlodzik, 1997), a transmembrane protein essential for Hedgehog signal transduction (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Conversely, ectopic expression of Hedgehog (Heberlein et al., 1995), or mutations in pka or ptc (Dominguez and Hafen, 1997; Ma and Moses, 1995; Pan and Rubin, 1995; Strutt and Mlodzik, 1995; Strutt et al., 1995; Wehrli and Tomlinson, 1995), which activate Hedgehog signal transduction, induce expression of photoreceptor-specific genes and set in motion waves of photoreceptor differentiation in the anterior part of the eye imaginal disc ahead of the endogenous morphogenetic furrow. These results indicate that Hedgehog expressed in photoreceptor cells signals anteriorly to drive the wave of photoreceptor differentiation.

In response to the Hedgehog signal, the bone morphogenetic protein (BMP) 2/4 homolog Decapentaplegic (Dpp) is expressed in cells of the morphogenetic furrow (Blackman et al., 1991; Heberlein et al., 1995; Masucci et al., 1990). Clonal loss of Dpp signal transduction due to mutations in thickveins (tkv), which encodes an essential receptor of Dpp (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Ruberte et al., 1995), retard photoreceptor differentiation (Burke and Basler, 1996; Greenwood and Struhl, 1999). Simultaneous loss of Hedgehog and Dpp signal transduction prevents photoreceptor differentiation, indicating that Hedgehog and Dpp signaling play a partially redundant role in this process (Curtiss and Mlodzik, 2000; Greenwood and Struhl, 1999).

Ectopic expression of Hedgehog also results in apical constriction and apicobasal shortening of cells, typical changes in cell shape that are associated with the normal morphogenetic furrow (Heberlein et al., 1995). Conversely, in the simultaneous absence of Hedgehog and Dpp signal transduction, cells fail to constrict apically, suggesting that Hedgehog and Dpp signaling promote apical constriction of cells within the morphogenetic furrow (Vrailas and Moses, 2006). However, whether Hedgehog and Dpp signaling are also necessary for the shortening of cells within the morphogenetic furrow is not known. Furthermore, the transcriptional targets of the Hedgehog and Dpp signaling pathways that induce these cell shape changes are elusive.

Here we show that Hedgehog and Dpp signal transduction promote the shortening of cells along their apicobasal axis within the morphogenetic furrow. Moreover, we provide evidence that Cad86C is a transcriptional target of these signaling pathways in the morphogenetic furrow that has the ability to organize elongated epithelial folds.

2. Results

2.1. Hedgehog and Dpp signaling are required for apicobasal shortening of cells in the morphogenetic furrow

Cells in the morphogenetic furrow are constricted at their apical side and shorter along their apicobasal axis. It was recently shown that the apical constriction of cells in the morphogenetic furrow depends on both Hedgehog and Dpp signal transduction (Vrailas and Moses, 2006). To test whether the cell shortening also depends on Hedgehog or Dpp signal transduction, we analyzed the shape of cells in smo^3 or tkv^{a12} mutant clones straddling the morphogenetic furrow. smo encodes a transmembrane protein essential for Hedgehog signal transduction (Alcedo et al., 1996; van den Heuvel and Ingham, 1996) and tkv encodes an essential receptor of Dpp (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Ruberte et al., 1995). Cell shape was analyzed by staining imaginal discs with rhodamine-phalloidin, which labels Filamentous (F)-actin, and recording confocal images in the plane of the epithelium (xy) and in cross sections (xz). Cross sections were either placed parallel, and within the morphogenetic furrow, or orthogonal to the morphogenetic furrow. Cell shapes were compared between mutant cells and neighboring control cells within the same imaginal disc. smo^3 mutant cells could apically constrict and shorten along their apicobasal axis, and could be part of a morphogenetic furrow, although the furrow in the smo^3 mutant clones was often more posterior in comparison to neighboring wild-type cells (Fig. 1C–E). This indicates that Hedgehog signal transduction is required for the timely progression of the morphogenetic furrow. Clones of cells mutant for tkv^{a12} could only be rarely recovered, as has been described previously (Vrailas and Moses, 2006 and references therein). tkv^{a12} mutant cells that were recovered, could apically constrict and shorten along their apicobasal axis, and formed an apparently normal morphogenetic furrow, which, however, was occasionally delayed compared to neighboring cells (Fig. 1F–H, and data not shown). Thus, neither Hedgehog signal transduction nor Dpp signal transduction appear to be essential for apical cell constriction and cell shortening. Therefore, we tested whether cells unable to transduce both the Hedgehog and the Dpp signal could form a morphogenetic furrow. smo^3 tkv^{a12} double mutant cells failed to constrict apically (Fig. 1I–L) and did not shorten along their apicobasal axis (Fig. 1M–O). We conclude that either Hedgehog or Dpp signal transduction is sufficient for apical constriction and apicobasal shortening of cells in the morphogenetic furrow. Moreover, these results indicate that apical cell constriction and cell shortening cannot be separated at the level of the signaling pathways, and that both Hedgehog and Dpp signaling pathways normally operate to promote these cell shape changes.

2.2. Cad86C is expressed in cells of the morphogenetic furrow

Ectopic expression of Ci^{PKA4}, an activated form of the Zn-finger containing protein Cubitus interruptus (Ci) (Methot and Basler, 2000), in clones of cells located anterior to the morphogenetic furrow resulted in apical cell constriction

(data not shown). Ci is an essential transcription factor of the Hedgehog signal transduction pathway (Dominguez et al., 1996), suggesting that Hedgehog signal transduction controls morphogenetic furrow formation by regulating gene expression. To identify genes regulated by Hedgehog signal transduction in eye imaginal discs, we analyzed the expression of selected genes encoding known or putative cell adhesion molecules by RNA in situ hybridization. One gene, *Cad86C* (Flybase *CG4655/CG4509*, Supplementary Fig. 1), was expressed in the broad region of the morphogenetic furrow and in clusters of cells posterior to the morphogenetic furrow, which presumably correspond to differentiating photoreceptor cells (Fig. 2A and B). In wing imaginal discs, Cad86C was detected uniformly and at low level throughout the epithelium (Supplementary Fig. 2). Cad86C, which has not been characterized in detail previously, is predicted to contain five extracellular cadherin (EC) domains and a single transmembrane domain and, thus, belongs to the family of Ca^{2+}-dependent cell–cell adhesion molecules (Supplementary Fig. 1). A consensus sequence for catenin-binding sites, as present in classical cadherins, was not identified. Moreover, expression of a HA-tagged version of Cad86C in wing imaginal disc cells did not obviously alter the subcellular distribution of Armadillo, the *Drosophila* homolog of β-catenin (Supplementary Fig. 3). Thus, Cad86C does not seem to bind to β-catenin, indicating that Cad86C is a non-classical cadherin.

To more precisely map the expression of Cad86C, and to analyze Cad86C protein in more detail, we raised two antibodies against Cad86C, M0881 and M0885 (see Section 4). Moreover, we generated two mutant alleles of *Cad86C*, termed *Cad86C[184A]* and *Cad86C[71C]*, by imprecise excision of an EP-element inserted in the first intron of *Cad86C* (see Section 4). In the *Cad86C[71C]* mutant allele, most of the predicted coding sequence for *Cad86C* is deleted whereas in the *Cad86C[184A]* mutant allele, the entire predicted coding sequence for *Cad86C* is absent. Flies homozygous mutant for either *Cad86C[184A]* or *Cad86C[71C]* are viable and fertile, indicating that *Cad86C* is not essential for development.

In the eye imaginal disc, Cad86C immunoreactivity was mainly detected in some cells of the morphogenetic furrow (Fig. 2C and E and Supplementary Fig. 2). Little or no immuno-reactivity was detected in eye imaginal discs from homozygous mutant *Cad86C[184A/184A]* larvae (Fig. 2D), indicating that the staining was specific for Cad86C. Specificity of the antibody was confirmed by Western blot analysis (Fig. 2G). To more precisely map the cells that express high levels of Cad86C protein, we recorded confocal images in cross sections (xz). Cad86C immunoreactivity was detected in cells of the anterior flank of the morphogenetic furrow (Fig. 2F). Little or no Cad86C immunoreactivity was detected in cells of the posterior flank of the morphogenetic furrow. Thus, we conclude that Cad86C protein distribution is asymmetric with respect to the morphogenetic furrow. Cad86C protein is present in cells at the leading (anterior) flank of the morphogenetic furrow, the cells that initially start to apically constrict and shorten.

2.3.   Cad86C localizes to the subapical region of cells

Next, we used our anti-Cad86C antibody to determine the subcellular localization of Cad86C protein within eye imaginal disc cells. In *Drosophila* epithelial cells, the lateral plasma membrane can be subdivided into three distinct domains (Knust and Bossinger, 2002). The most apical domain of the lateral plasma membrane, the subapical region, is followed by the zonula adherens, and then basally by the septate junction. Using the antibody against Cad86C, strong immunoreactivity was detected in columnar cells apical to the zonula adherens, as marked by DE-cadherin, and partially overlapped with a marker for the subapical region, DPatj (Bhat et al., 1999), in wild-type eye imaginal discs (Fig. 2E and F). We conclude that Cad86C localizes to the subapical region of eye imaginal disc cells within the morphogenetic furrow.

2.4.   Hedgehog signal transduction induces Cad86C expression

We have shown above that Cad86C localizes to the subapical region of cells located on the anterior flank of the morphogenetic furrow. Hedgehog is expressed in differentiated cells posterior to the morphogenetic furrow and spreads anteriorly to induce the expression of target genes,

Fig. 1 – Hedgehog and Dpp signal transduction are required for apicobasal shortening of cells within the morphogenetic furrow. (A) Scheme of an eye imaginal disc. The morphogenetic furrow (MF) moves from posterior to anterior across the eye imaginal disc. Cells differentiate as they leave the morphogenetic furrow. Differentiated cells express Hedgehog. Hedgehog signals to the anterior and induces expression of Dpp in the morphogenetic furrow. (B) Scheme of a cross section through the eye imaginal disc at the position indicated by the red line in (A). The columnar cells of the eye imaginal disc epithelium are apically constricted and shorter within the morphogenetic furrow. Dark blue lines indicate zonula adherens. A layer of squamous cells, the peripodial membrane (PM), overlies the columnar cells. (C–O‴) Clones of cells homozygous mutant for (C–E′) smo[3], (F–H′) tkv[a12], and (I–O‴) smo[3] tkv[a12] are marked by the absence of GFP staining (green). Rhodamine-phalloidin staining is shown in red and E-cadherin is shown in white. (D–E′), (G–H′), and (M–O‴) are cross sections. The positions of the cross sections are indicated by the dotted lines in the corresponding xy sections. The letters next to the dotted lines refer to the panel in which the cross section is shown. (J–L) Magnified views of (J) cells of the morphogenetic furrow, (K) cells anterior to the morphogenetic furrow, and (L) smo[3] tkv[a12] cells straddling the morphogenetic furrow at the apicobasal level of E-cadherin. The corresponding areas are boxed in (I). Cells within tkv[a12] or smo[3] mutant clones can still apically constrict (arrows in C and F) and form a morphogenetic furrow. smo[3] tkv[a12] double mutant cells fail to apically constrict and fail to form a morphogenetic furrow. In these and all subsequent figures, images are xy images, except noted otherwise. In xy images, posterior is shown to the right. In optical cross sections (xz), apical of the columnar cells is to the top. Scale bars: 10 μm.

including Dpp, in cells anterior to, and within, the morphogenetic furrow (Heberlein et al., 1993, 1995; Ma et al., 1993). Transduction of the Hedgehog signal prevents the proteolytic cleavage of Ci and results in the formation of a full-length form of Ci (Ci-155) (Aza-Blanc et al., 1997). To test whether the Hedgehog signal induces the expression of Cad86C in the morphogenetic furrow, we first stained eye imaginal discs with the anti-Cad86C antiserum and an antibody detecting Ci-155. Ci-155 levels were elevated in cells on the anterior flank of the morphogenetic furrow compared to cells on the posterior side (Fig. 3A and B), indicating that the Hedgehog signal is transduced mainly in cells of the anterior flank of the morphogenetic furrow. Cad86C staining was detected in cells expressing high levels of Ci-
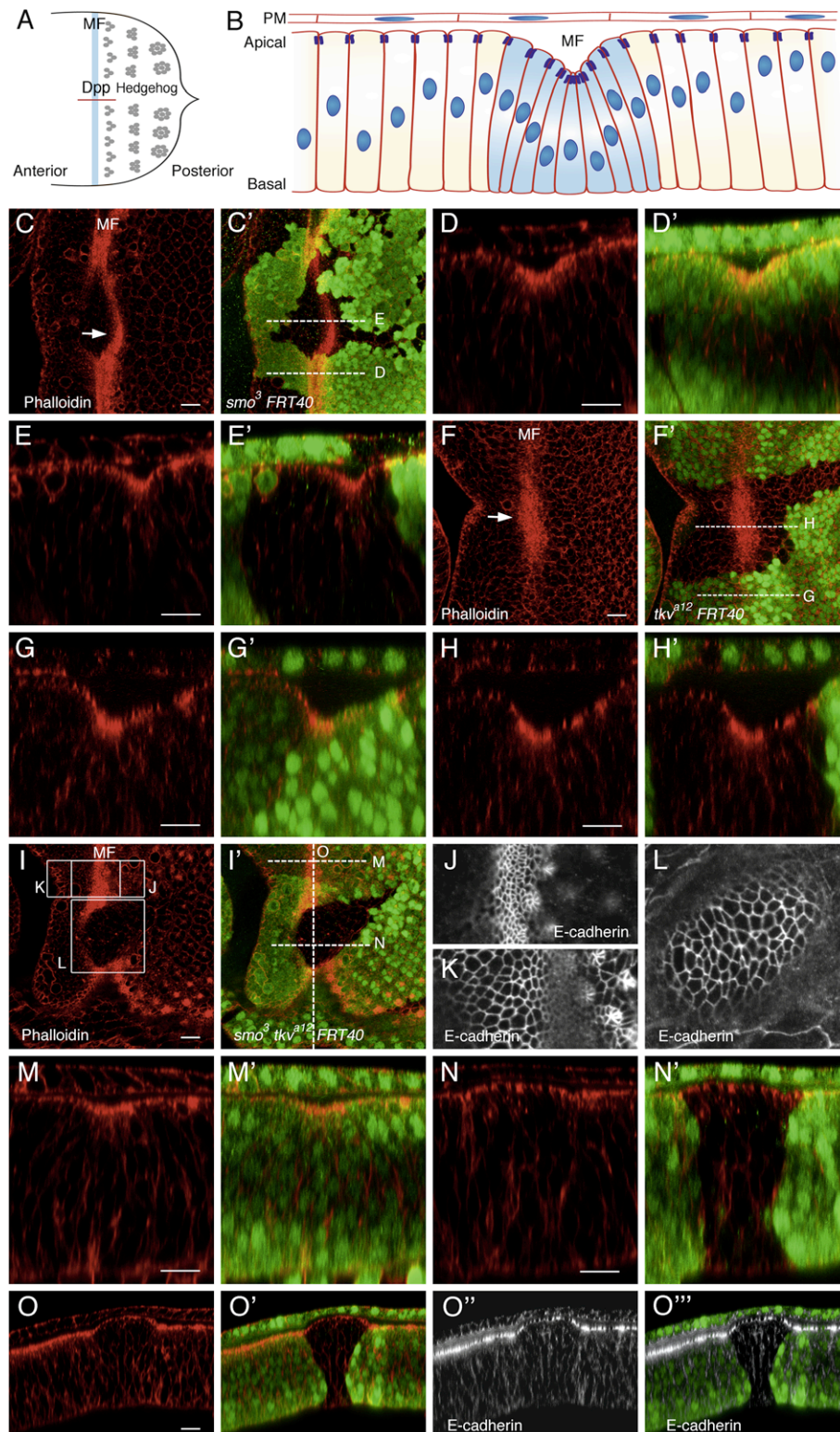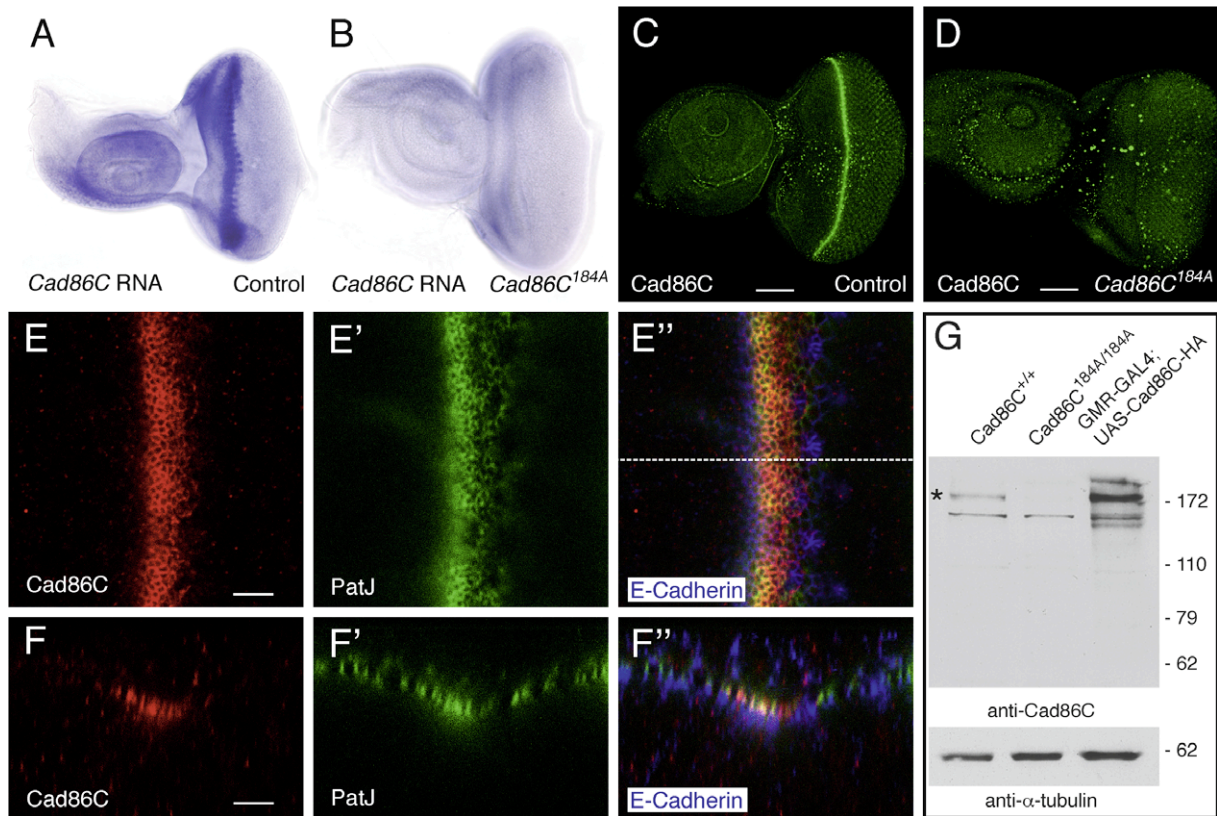
Fig. 2 – Cad86C is expressed in cells of the leading flank of the morphogenetic furrow and localizes to the subapical region. (A) A control eye imaginal disc hybridized with a *Cad86C*-specific RNA probe. A hybridization signal is detected in the region of the morphogenetic furrow. (B) An eye imaginal disc of a homozygous mutant *Cad86C*[184A/184A] larva hybridized with a *Cad86C*-specific RNA probe. Little or no hybridization signal is detected. (C) A control eye imaginal disc stained with an affinity purified anti-Cad86C antibody (MO881). Strong immunoreactivity is detected in cells of the morphogenetic furrow. (D) An eye imaginal disc of a homozygous mutant *Cad86C*[184A/184A] larva stained with an affinity purified anti-Cad86C antibody (MO881). Little or no immunoreactivity is detected. (E–E″) A magnified view of a control eye imaginal disc stained for Cad86C (E, red), PatJ (E′, green), and E-cadherin (blue). A merge is shown in (E″). (F) A cross section of the eye imaginal disc shown in (E). The position of the cross section is indicated by a dotted line in (E″). Cad86C co-localizes with PatJ to the subapical region of columnar cells. (G) Extracts of eye imaginal discs collected from control (*Cad86C*^{+/+}, lane 1) and homozygous mutant *Cad86C*[184A/184A] (lane 2) larvae, and from larvae overexpressing Cad86C-HA (GMR-GAL4; UAS-*Cad86C*-HA) (lane 3) were probed with an affinity purified anti-Cad86C antibody (M0881). A protein of ~180 kDa (asterisk) was detected in extracts of wild-type (lane 1) and Cad86C-HA overexpressing (lane 3) larvae. No immunoreactivity against this ~180 kDa protein was detected in extracts from mutant larvae (lane 2). Immunoreactivity against the ~180 kDa protein was increased when Cad86C is overexpressed. Subsequent blotting with an anti-α-tubulin antibody (lower panel) indicated that equal amounts of protein were loaded in each lane. The apparent molecular weight of 180 kDa is smaller than the predicted size of the conceptual translation of the *Cad86C* transcript (220 kDa), indicating that the Cad86C protein might be posttranslationally processed. This processing might include the proteolytic cleavage of a putative precursor Cad86C protein into the mature form, as has been shown for other cadherins (Ozawa and Kemler, 1990 and references therein). Molecular weights are indicated to the right (in kDa). Scale bars: (C and D) 50 μm; (E and F) 5 μm.

155, providing a first indication that Hedgehog signaling regulates Cad86C expression.

To corroborate these results, we activated the Hedgehog signal transduction pathway in marked clones of cells by expressing the constitutively active form of Ci, Ci[PKA4], and analyzed the expression of Cad86C in eye imaginal discs. Clones of cells located anterior, and close to the morphogenetic furrow, showed elevated levels of Cad86C (Fig. 3C), indicating that Hedgehog signaling induces Cad86C expression.

2.5. Dpp signal transduction induces Cad86C expression in the morphogenetic furrow

To test whether Dpp signaling could also increase expression of Cad86C in the eye imaginal disc, we activated the Dpp signal transduction pathway in marked clones of cells by expressing a constitutively active form of the Dpp receptor Thickveins, Tkv[Q253D] (Lecuit et al., 1996; Nellen et al., 1996), and analyzed the expression of Cad86C. Tkv[Q253D] expression resulted in a moderate in-
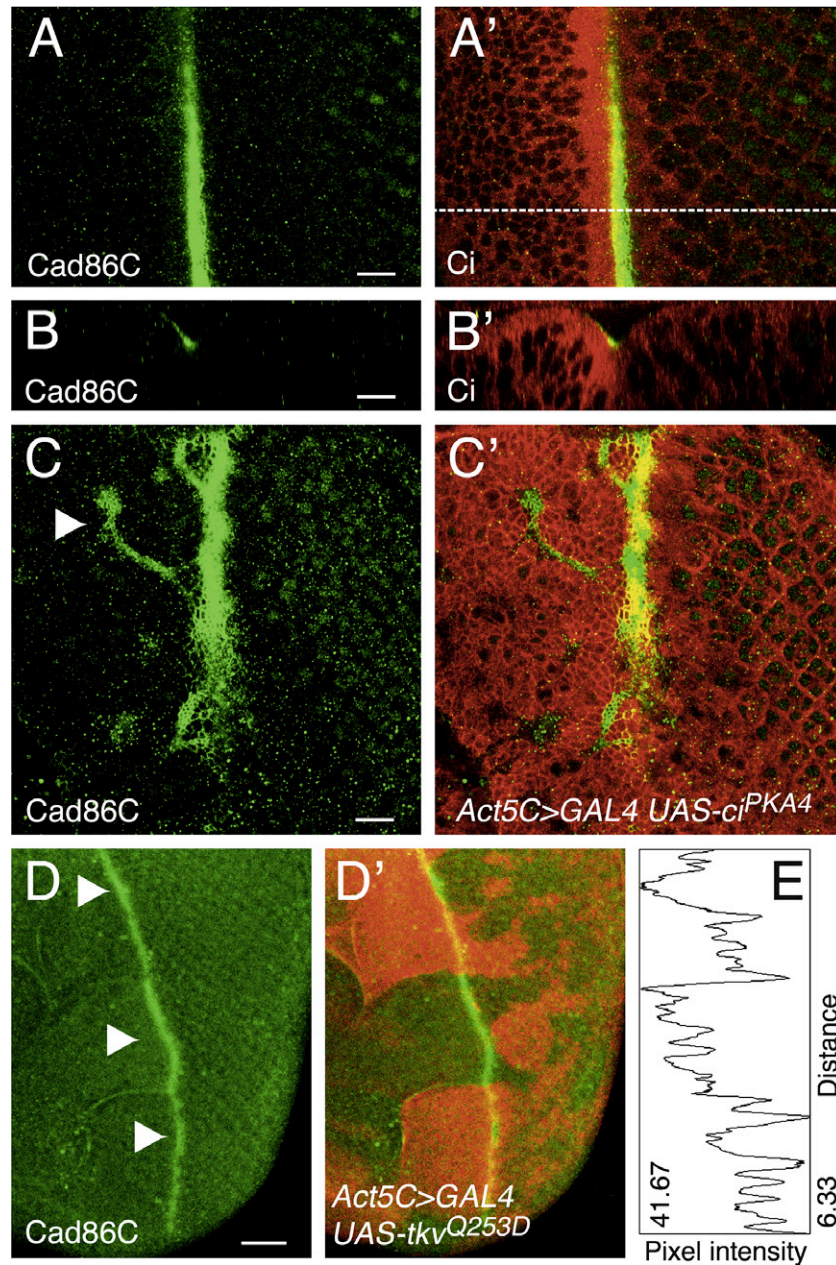
Fig. 3 – Cad86C expression in the morphogenetic furrow is induced by Hedgehog and Dpp signaling. (A and A′) A magnified view of an eye imaginal disc stained for Cad86C (MO881) (green) and Ci (A′, red). (B and B′) A cross section of the eye imaginal disc shown in (A). The position of the cross section is indicated by a dotted line in (A′). Cad86C localizes to cells of the anterior flank of the morphogenetic furrow that show elevated levels of Ci-155 staining. (C and C′) An eye imaginal disc expressing Ci^{PKA4} for 18 h in clones of cells (Act5C>GAL4, UAS-Ci^{PKA4}) marked by the absence of CD2 (red) and stained for Cad86C (MO881, green). Cad86C levels are increased inside clones of cells located anterior and close to the morphogenetic furrow (arrow). (D and D′) An eye imaginal disc expressing Tkv^{Q253D} for three days in clones of cells (Act5C>GAL4, UAS-tkv^{Q253D}) marked by the absence of CD2 (red) and stained for Cad86C (MO881, green). Cad86C levels are moderately increased in cells of the morphogenetic furrow (arrows). (E) Pixel intensity plot of Cad86C staining (arbitrary units) of (D). Scale bars: (A–C) 10 μm; (D) 25 μm.

crease of Cad86C within the morphogenetic furrow, but not elsewhere within the eye imaginal disc (Fig. 3D and E). Cad86C expression was not obviously different in control clones (Supplementary Fig. 4). We conclude that Dpp signaling induces Cad86C expression in cells of the morphogenetic furrow.

2.6. Hedgehog and Dpp signal transduction is required for Cad86C expression

To test whether Hedgehog and Dpp signal transduction is required for Cad86C expression, we analyzed smo^3 mutant clones and tkv^{a12} mutant clones straddling the mor-

phogenetic furrow for the presence of Cad86C. Cad86C immunoreactivity was detected in these smo^3 mutant and tkv^{a12} mutant clones (Fig. 4A and B) indicating that neither Smo-mediated Hedgehog signal transduction nor Tkv-mediated Dpp signal transduction is essential for the expression of Cad86C in cells of the morphogenetic furrow.

To test whether Dpp signaling and Hedgehog signaling act redundantly to induce expression of Cad86C, we generated and analyzed clones of cells mutant for both smo^3 and tkv^{a12}
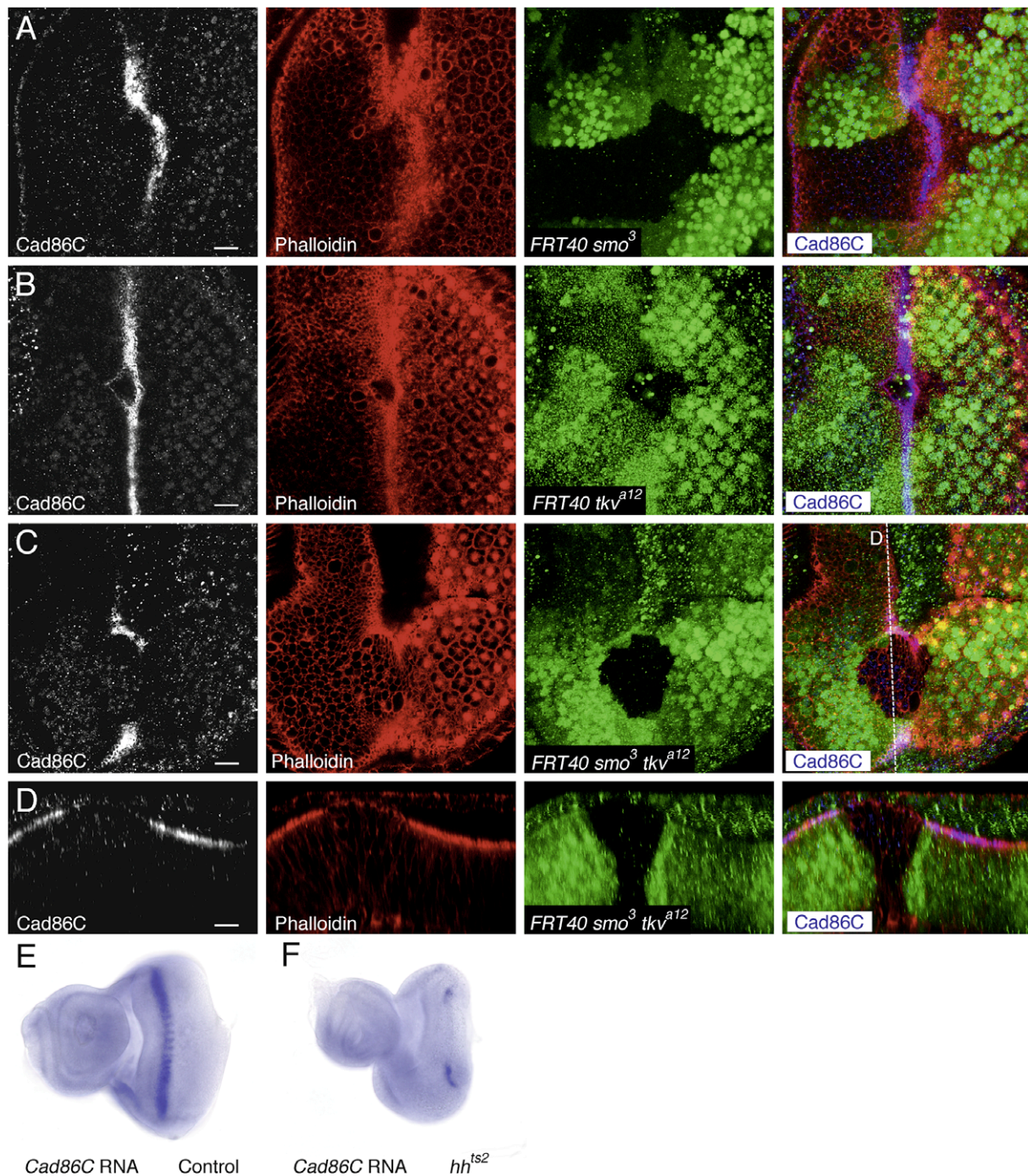

Fig. 4 – Hedgehog and Dpp signaling are required for Cad86C expression in the morphogenetic furrow. (A–D) Clones of cells homozygous mutant for (A) smo^3, (B) tkv^{a12}, and (C and D) smo^3 tkv^{a12} are marked by the absence of GFP staining (green). Cad86C (white) and rhodamine-phalloidin (red) staining are shown. (D) A cross section of the eye imaginal disc shown in (C) at the position indicated by a dotted line. tkv^{a12} or smo^3 mutant cells express Cad86C. Cad86C staining is highly reduced in smo^3 tkv^{a12} double mutant cells. (E and F) Eye imaginal disc of (E) control $hh^{ts/+}$ larvae and (F) mutant $hh^{ts2/ts2}$ larvae raised for 2 days at 29 °C and hybridized with a Cad86C-specific RNA probe. The Cad86C hybridization signal is highly reduced in $hh^{ts2/ts2}$ mutants. Scale bars: (A–D) 10 μm.

straddling the normal position of the morphogenetic furrow. Cad86C immunoreactivity was highly reduced in these clones (Fig. 4C and D). E-Cadherin was still present in smo^3 tkv^{a12} double mutant clones straddling the normal position of the morphogenetic furrow, albeit at a somewhat reduced level (Fig. 1L and O), indicating that the failure of smo^3 tkv^{a12} double mutant cells to constrict apically cannot account for the loss of Cad86C immunoreactivity. We conclude that both Dpp and Hedgehog signal transduction promote Cad86C protein expression in the morphogenetic furrow and that Cad86C protein fails to be highly expressed only when both signal transduction pathways are inactivated.

To test whether the Hedgehog and Dpp signal transduction pathways promote Cad86C protein expression by inducing Cad86C transcription or by posttranscriptional regulation, we analyzed the levels of Cad86C transcript in eye imaginal discs homozygous mutant for a temperature-sensitive allele of hedgehog, hh^{ts2}, at restrictive temperature by RNA in situ hybridization. Under these experimental conditions, progression of the morphogenetic furrow is halted and the expression of dpp is highly reduced within the morphogenetic furrow (Ma et al., 1993) (data not shown). As shown in Fig. 4E and F, expression of Cad86C was abolished in the center of the eye imaginal disc and some variable residual expression remained towards the edges. We conclude that the Hedgehog signal transduction pathway is required for Cad86C transcription within cells of the morphogenetic furrow.

2.7. Ectopic Cad86C expression can form elongated epithelial folds

$Cad86C^{184A}$ as well as $Cad86C^{71C}$ homozygous mutant cells were able to form an apparently normal morphogenetic furrow (Supplementary Fig. 5). Moreover, photoreceptor differentiation appeared to be normal in Cad86C mutant eye imaginal discs, as analyzed by the neuronal marker Elav (data not shown). It is conceivable that Hedgehog and Dpp signal transduction promote the progression of the morphogenetic furrow through induction of genes in addition to Cad86C. The role of Cad86C in morphogenetic furrow formation may therefore be masked by redundancy with another protein (s). Non-classical cadherins have been shown previously to genetically interact with E-cadherin during embryonic development (Lovegrove et al., 2006). However, Cad86C did not appear to genetically interact with E-cadherin during eye development, since cells doubly mutant for Cad86 and shg, which encodes E-cadherin, formed an apparently normal morphogenetic furrow (Supplementary Fig. 6). To test whether Cad86C can promote the formation of a morphogenetic furrow, we ectopically expressed an HA-tagged version of Cad86C, Cad86C-HA, using the GAL4-UAS system in clones of cells within the eye imaginal disc. The shape of cells was analyzed by xy and xz-confocal scanning. Clones of cells sometimes (16%, $n = 76$ clones) tended to have an unusual shape in that they were highly elongated (Fig. 5A and B). Control clones were not highly elongated and showed a more irregular shape (data not shown). xz-Scans revealed that cells of highly elongated clones expressing Cad86C-HA were shorter along their apicobasal axis, were apically constricted, and were part of an epithelial fold (Fig. 5C; data not shown). The

epithelial folds also contained neighboring wild-type cells, indicating that the Cad86C-HA expressing cells pulled some wild-type cells into the fold. Thus, Cad86C-HA is able to induce elongated epithelial folds within the eye imaginal disc.

The ability of Cad86C-HA to induce elongated epithelial folds could depend on additional eye imaginal disc-specific molecules and, therefore, could be restricted to the eye imaginal disc. Alternatively, the ability of Cad86C-HA to induce epithelial folds could be widespread among different epithelia. To distinguish between these possibilities, we generated and analyzed clones of cells expressing Cad86C-HA in wing imaginal discs. In contrast to the control clones, clones in wing imaginal discs expressing Cad86C-HA sometimes (17%, $n = 84$ clones) had highly elongated shapes (Fig. 5D; data not shown). In these clones, cells expressing Cad86C-HA were shorter along their apicobasal axis compared to neighboring control cells, were apically constricted, and were part of an epithelial fold (Fig. 5E–I). Similar to clones in the eye imaginal disc, also wild-type cells adjacent to the Cad86C-HA expressing cells formed part of the epithelial folds. Expression of E-cadherin or Cad99C, two other members of the cadherin superfamily, in clones of cells did not result in highly elongated clone shapes that were associated with epithelial folds (D'Alterio et al., 2005; Dahmann and Basler, 2000) (data not shown). We conclude that the ability of Cad86C to induce elongated epithelial folds is not limited to eye imaginal discs, but appears widespread.

2.8. Epithelial folds form at the border between Cad86C-HA expressing cells and control cells

Expression of Cad86C-HA in clones of cells could cell-autonomously lead to cell shortening and, thus, to epithelial fold formation. Alternatively, cell shortening could be a consequence of the apposition of Cad86C-HA-expressing cells and control cells. To distinguish between these two possibilities, we expressed Cad86C-HA in a large area of cells, the dorsal compartment of wing imaginal discs, and analyzed cell shape. If Cad86C-HA expression cell-autonomously leads to cell shortening, all cells of the dorsal compartment are expected to be short. On the other hand, if the apposition of Cad86C-HA-expressing cells and control cells is responsible for shortening of cells, then only cells at the dorsal–ventral compartment boundary should be short whereas cells within the center of the dorsal compartment should be of normal length. The apicobasal length of control cells, and cells expressing Cad86C-HA in the center of the dorsal compartment, was indistinguishable (Fig. 6). In contrast, at the dorsal–ventral compartment boundary, both ventral control cells and dorsal cells expressing Cad86C-HA had sometimes a highly decreased cell length and were part of a deep epithelial fold. Thus, expression of Cad86C-HA does not result in a cell autonomous cell shortening. Cell shortening and epithelial folding are rather consequences of the apposition of Cad86C-HA expressing cells and control cells.

2.9. The extracellular and transmembrane regions of Cad86C are sufficient to induce elongated epithelial folds

Cad86C is a transmembrane protein consisting of an extracellular region composed of five cadherin domains and an

intracellular region. To test whether the extracellular region or the intracellular region is sufficient to induce epithelial folding, we generated HA-tagged constructs lacking either the intracellular region of Cad86C (Cad86C-EXTRA-HA) or most of the extracellular region of Cad86C (Cad86C-INTRA HA, see Section 4). These truncated proteins were then expressed using the GAL4-UAS system in clones of cells within the eye imaginal disc. Similar to Cad86C-HA, both Cad86C-INTRA-HA and Cad86C-EXTRA-HA were enriched at the apical side of cells as revealed by an antibody directed against the HA tag (Fig. 7B and D). Epithelial folds were not observed when Cad86C-INTRA-HA was expressed (Fig. 7A and B). Moreover, expression of a fusion protein comprising the extracellular region of E-cadherin and both the transmembrane and intracellular region of Cad86C failed to induce epithelial folds (data not shown). In contrast, expression of Cad86C-EXTRA HA resulted in elongated epithelial folds similar to expression of the full-length Cad86C-HA protein (Fig. 7C and D). We conclude that the extracellular region and transmembrane domain of Cad86C are sufficient to induce elongated epithelial folds.

2.10. Homophilic binding of Cad86C in trans is not required for Cad86C localization on plasma membranes

The experiments described above indicate an important role for the extracellular cadherin repeats of Cad86C-HA to promote cell shortening. Cadherin repeats can confer homophilic or heterophilic binding to other cadherin molecules *in trans*. One way to distinguish homophilic from heterophilic binding in vivo is to analyze the distribution of cadherins in wild-type cells facing cells lacking that particular cadherin. E-cadherin, which is known to interact homophilically, for example, is highly reduced at plasma membranes of control cells facing cells lacking E-cadherin (Wei et al., 2005) (data not shown). To test whether Cad86C binds homophilically or heterophilically, we generated $Cad86C^{71C}$ homozygous mutant clones of cells straddling the morphogenetic furrow and analyzed the distribution of Cad86C in wild-type cells facing mutant cells. As shown in Fig. 7E, Cad86C protein was not noticeably decreased on cell membranes facing mutant cells.

These data suggest that Cad86C acts as a heterophilic binding molecule.

2.11. Expression of Cad86C-HA promotes shortening of Hedgehog and Dpp signaling compromised cells straddling the morphogenetic furrow

We have shown above that, within the morphogenetic furrow, cells lacking Hedgehog and Dpp signal transduction express Cad86C at a highly reduced level and fail to shorten along their apicobasal axis. To test whether Cad86C, in wild-type, can mediate the shortening of cells in the morphogenetic furrow in response to Hedgehog and Dpp signal transduction, we expressed Cad86C-HA in GFP-marked smo^3 tkv^{a12} double mutant clones and analyzed the shape of mutant cells located in the region of the morphogenetic furrow. GFP-marked control smo^3 tkv^{a12} double mutant cells failed to shorten along their apicobasal axis (Fig. 8A and B). In 7 out of 12 GFP-marked smo^3 tkv^{a12} double mutant clones expressing Cad86C-HA, cells failed to shorten along their apical–basal axis (data not shown). In contrast, in the remaining five clones cells formed an epithelial invagination. Cells at the center of this invagination were much shorter along their apicobasal axis compared to control smo^3 tkv^{a12} double mutant cells, or wild-type cells within the morphogenetic furrow (Fig. 8C–F). Thus, expression of Cad86C-HA can promote the shortening of Hedgehog and Dpp signaling compromised cells straddling the morphogenetic furrow. This result is consistent with the view that Cad86C, in part, mediates the shortening of cells within the morphogenetic furrow in response to Hedgehog and Dpp signaling.

3. Discussion

The progression of the morphogenetic furrow provides an example of a developmentally regulated cell shape change. Here we have studied the signaling pathways that regulate this cell shape change and have identified a transcriptional target of these pathways. We found that the Hedgehog and Dpp signaling pathways both promote the shape change of cells that normally occurs in the morphogenetic furrow.

Fig. 5 – Ectopic Cad86C-HA expression can form elongated epithelial folds. (A) An eye imaginal disc expressing Cad86C-HA in clones of cells (*Act5C>GAL4, UAS-Cad86C-HA*), and stained for HA (green) and rhodamine-phalloidin (red). Arrowheads indicate the position of the morphogenetic furrow. The elongated epithelial fold anterior to the morphogenetic furrow is also associated with a clone, however, Cad86C-HA is out of the focal plane and, therefore, not visable. (B) A magnified view of the area boxed in (A). (C) A cross section of the eye imaginal disc shown in (B). The position of the cross section is indicated by a dotted line in (B). Cells of the clone are associated with an ectopic apical fold (arrow). (D) A wing imaginal disc expressing Cad86C-HA in clones of cells (*Act5C>GAL4, UAS-Cad86C-HA*), and stained for Cad86C (green) and rhodamine-phalloidin (red). (E) A magnified view of the area boxed in (D). (F) A cross section of the wing imaginal disc shown in (E). The position of the cross section is indicated by a dotted line in (E). Cells of the clone are associated with an ectopic apical fold (arrow). (G–I) A wing imaginal disc expressing Cad86C-HA in clones of cells (*Act5C>GAL4, UAS-Cad86C-HA*), and stained for E-cadherin (red) and Cad86C (G′, green). (G) Cross section. Pairs of arrows and arrowheads point to the distance between two E-cadherin-labelled zonula adherens of control cells and cells expressing Cad86C-HA, respectively. (H and I) xy sections of the wing imaginal disc shown in (G) at the indicated positions (dotted lines). The apical circumferences of cells expressing Cad86C-HA (I, arrows) are reduced compared to control cells (H). Scale bars: (B, C, E–I) 10 μm; (A and D) 100 μm.

Moreover, we have identified a gene, *Cad86C*, which is expressed in cells of the morphogenetic furrow and provide evidence that expression of this gene is regulated by both Hedgehog and Dpp signaling. Finally, we show that Cad86C possesses, among known cadherins, an unique activity to organize elongated epithelial folds. Our data suggest that Cad86C is a transcriptional target gene of Hedgehog and Dpp in the morphogenetic furrow. Furthermore, our data are consistent with the notion that Cad86C might be one effector that acts downstream of Hedgehog and Dpp signaling to help

execute the cell shape changes associated with the progression of the morphogenetic furrow.

3.1. Hedgehog and Dpp signaling regulate Cad86C expression in cells of the morphogenetic furrow

Our conclusion that Cad86C expression in the morphogenetic furrow is regulated by Hedgehog and Dpp signal transduction is derived from the analysis of loss-of-function mutants in these signaling pathways and from the ectopic
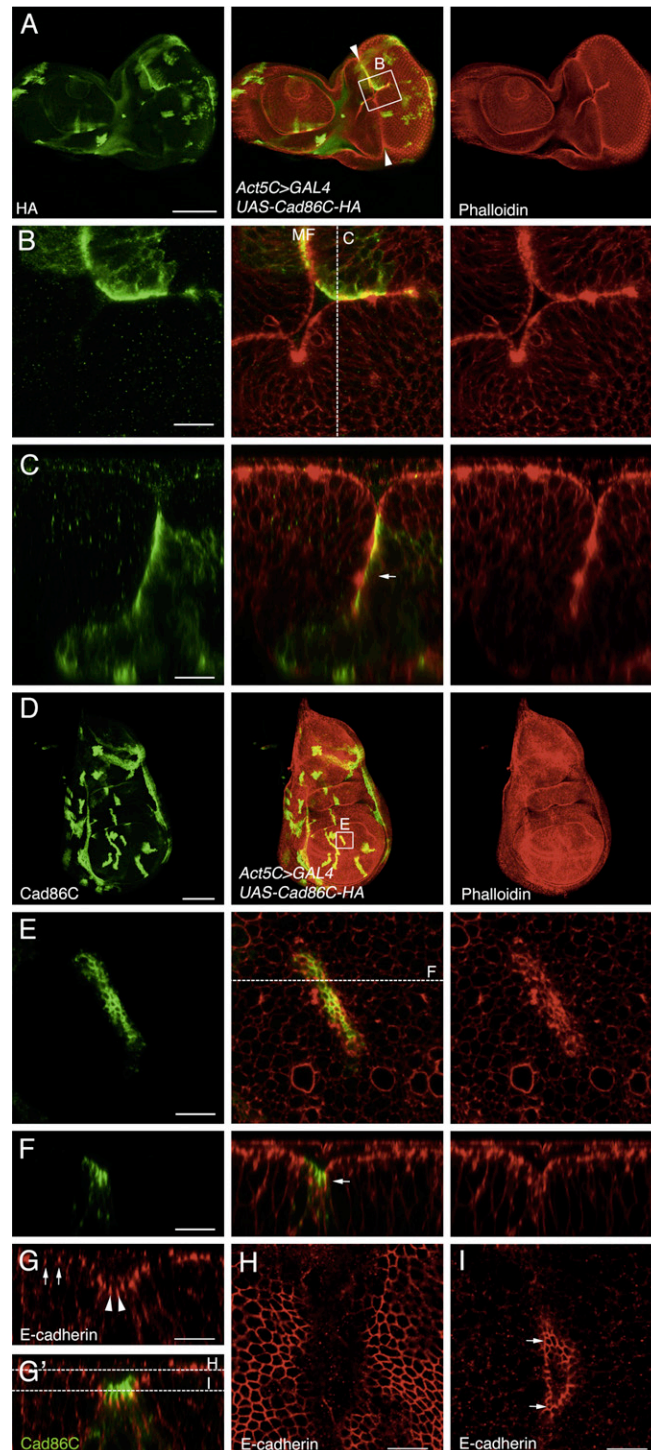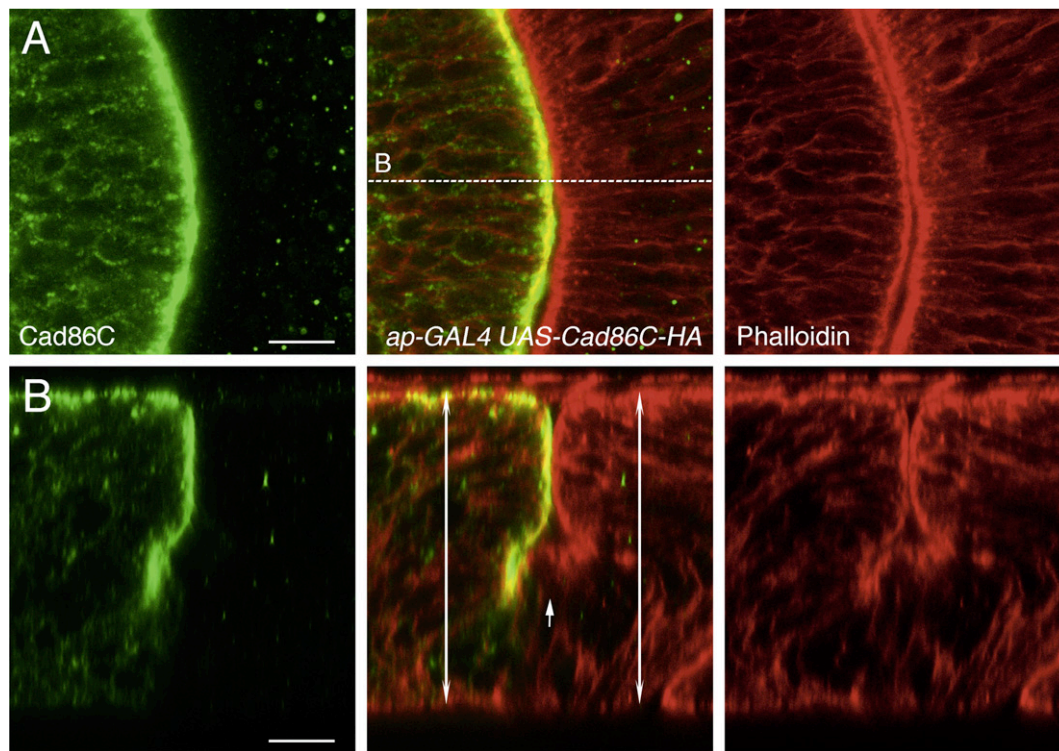
Fig. 6 – Epithelial folds form at the border between Cad86C-HA expressing cells and control cells. (A) A wing imaginal disc expressing Cad86C-HA in the dorsal compartment (*ap-GAL4, UAS-Cad86C-HA*), and stained for Cad86C (green) and rhodamine-phalloidin (red). Dorsal is shown to the left. (B) A cross section of the wing imaginal disc shown in (A). The position of the cross section is indicated by a dotted line in (A). The columnar epithelium of the dorsal and ventral compartments is of similar height (double-sided arrows). Cells at the dorsal–ventral compartment boundary have a decreased length and are part of an epithelial fold (arrow). Scale bars: 10 μm.

activation of these signaling pathways through expression of activated components. We found that the level of Cad86C protein is highly reduced in smo^3 tkv^{a12} clones straddling the normal position of the morphogenetic furrow, whereas Cad86C protein is still detectable in smo^3 or tkv^{a12} single mutant clones. Conversely, expression of a constitutively active form of the Hedgehog-regulated transcription factor Ci, Ci^{PKA4}, or a constitutively active form of the Dpp receptor Thickveins, Tkv^{Q253D}, resulted in increased levels of Cad86C protein. Two observations indicate that Hedgehog and Dpp signal transduction regulate the expression of Cad86C mainly at a transcriptional level. First, the abundance of *Cad86C* RNA is highly increased in the morphogenetic furrow of wild-type eye imaginal discs and, second, *Cad86C* RNA is highly reduced when *hedgehog* activity (and Dpp expression) is impaired in hh^{ts2} mutant eye imaginal discs. We have identified, in the first intron of *Cad86C*, a cluster of three putative Ci binding sites based on their sequence similarity to the Gli/Ci consensus binding sequence (Kinzler and Vogelstein, 1990) (Supplementary Fig. 7A). This provides a first indication that *Cad86C* might be a direct transcriptional target of the Hedgehog signaling pathway. In *Cad86C^{71C}* mutants, in which these putative Ci binding sites are deleted, *Cad86C* RNA appears to be normally expressed in the morphogenetic furrow (Supplementary Fig. 7B and C), demonstrating that these sites are not essential for *Cad86C* expression in the morphogenetic furrow. This is consistent with our finding that the Hedgehog

signal transduction pathway is not essential for Cad86C expression in cells of the morphogenetic furrow and, that in its absence, the Dpp signaling pathway can promote expression of Cad86C. Cad86C expression, in addition, might be controlled also at a posttranscriptional level, since *Cad86C* RNA, but not Cad86C protein, is detected in some cells posterior to the morphogenetic furrow.

3.2. *Cad86C and the formation of epithelial folds*

In contrast to other known cadherins, Cad86C possesses an unique activity to organize elongated folds in epithelia. This activity appears to be mediated by the cadherin repeats, since expression of a deletion variant of Cad86C, Cad86C-EXTRA-HA, in which the intracellular region is missing, still induces epithelial folds. Since cadherin repeats mediate the binding between cadherin molecules, we speculate that expression of Cad86C-HA promotes epithelial folding through its interaction with a cadherin. We do not find evidence that Cad86C interacts homophilically in cells of the morphogenetic furrow. Cad86C might, therefore, interact with a different kind of cadherin to promote epithelial folding. Candidates for Cad86C-interacting cadherins include the non-classical cadherins Cad74A, Cad88C, and Cad96Cb, which, during embryonic spiracle development, are expressed in sub-sets of cells adjacent to Cad86C (Lovegrove et al., 2006). Among these three cadherins, we find that *Cad88C*
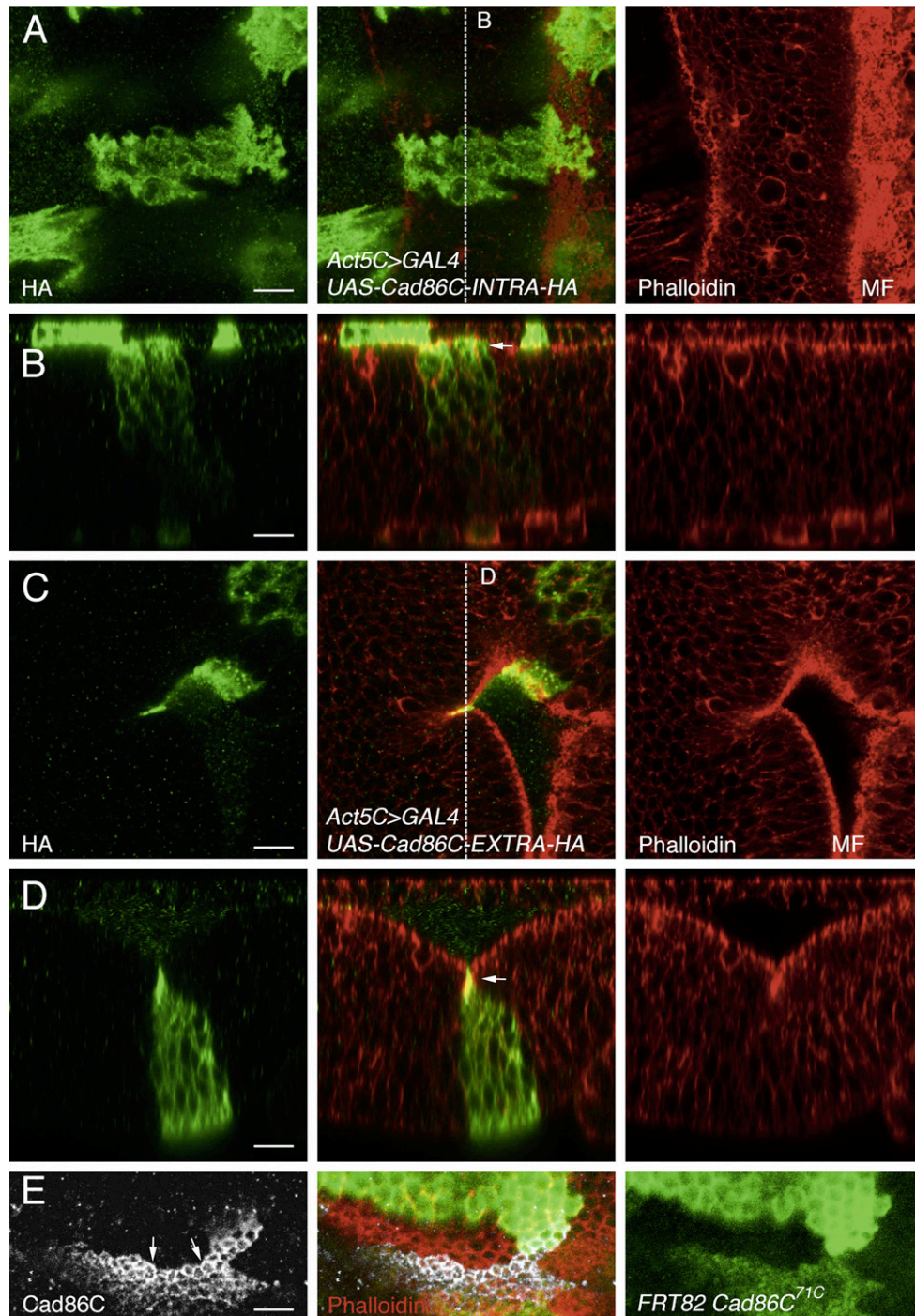
Fig. 7 – Expression of Cad86C-EXTRA-HA is sufficient to induce epithelial folds. (A) An eye imaginal disc expressing Cad86C-INTRA-HA in clones of cells (*Act5C>GAL4, UAS-Cad86C-INTRA-HA*), and stained for HA (green) and rhodamine-phalloidin (red). (B) A cross section of the eye imaginal disc shown in (A). The position of the cross section is indicated by a dotted line in (A). Cad86C-INTRA-HA localizes to the apical side of columnar cells (arrow). No epithelial folds associated with these clones were observed. In addition to the clone in the columnar epithelium, two clones are present in the peripodial membrane. (C) An eye imaginal disc expressing Cad86C-EXTRA-HA in clones of cells (*Act5C>GAL4, UAS-Cad86C-EXTRA-HA*), and stained for HA (green) and rhodamine-phalloidin (red). (D) A cross section of the eye imaginal disc shown in (C). The position of the cross section is indicated by a dotted line in (C). Cad86C-EXTRA-HA localizes to the apical side of columnar cells (arrow). Cells of the clone are associated with an ectopic apical fold (arrow). (E) Clones of cells homozygous mutant for *Cad86C^{71C}* are marked by the absence of CD2 staining (green). Cad86C (white) and rhodamine-phalloidin (red) staining are shown. Cad86C protein is present on cell membranes of control cells facing *Cad86C^{71C}* mutant cells (arrows). Scale bars: (A–D) 10 μm; (E) 5 μm.

is expressed in a complementary pattern to *Cad86C* (Supplementary Fig. 2). However, adult flies homozygous mutant for *Cad86C* and *Cad88C* had an apparently normal eye size (data not shown), indicating that Cad88C is, if at all, not an essential interacting partner for Cad86C during morphogenetic furrow progression.

Cad86C-HA induces epithelial folding non-cell-autonomously, indicating that an imbalance in the expression level of Cad86C between neighboring cells might result in cell shortening. We note, however, that *Cad86C^{184A}* mutant clones in the wing imaginal disc and eye imaginal disc are not associated with epithelial folds (data not shown), perhaps because the absolute difference in Cad86C expression between mutant cells and neighboring control cells is only modest.

Similar to the expression of Cad86C-HA, expression of an activated form of the regulatory light chain of non-muscle
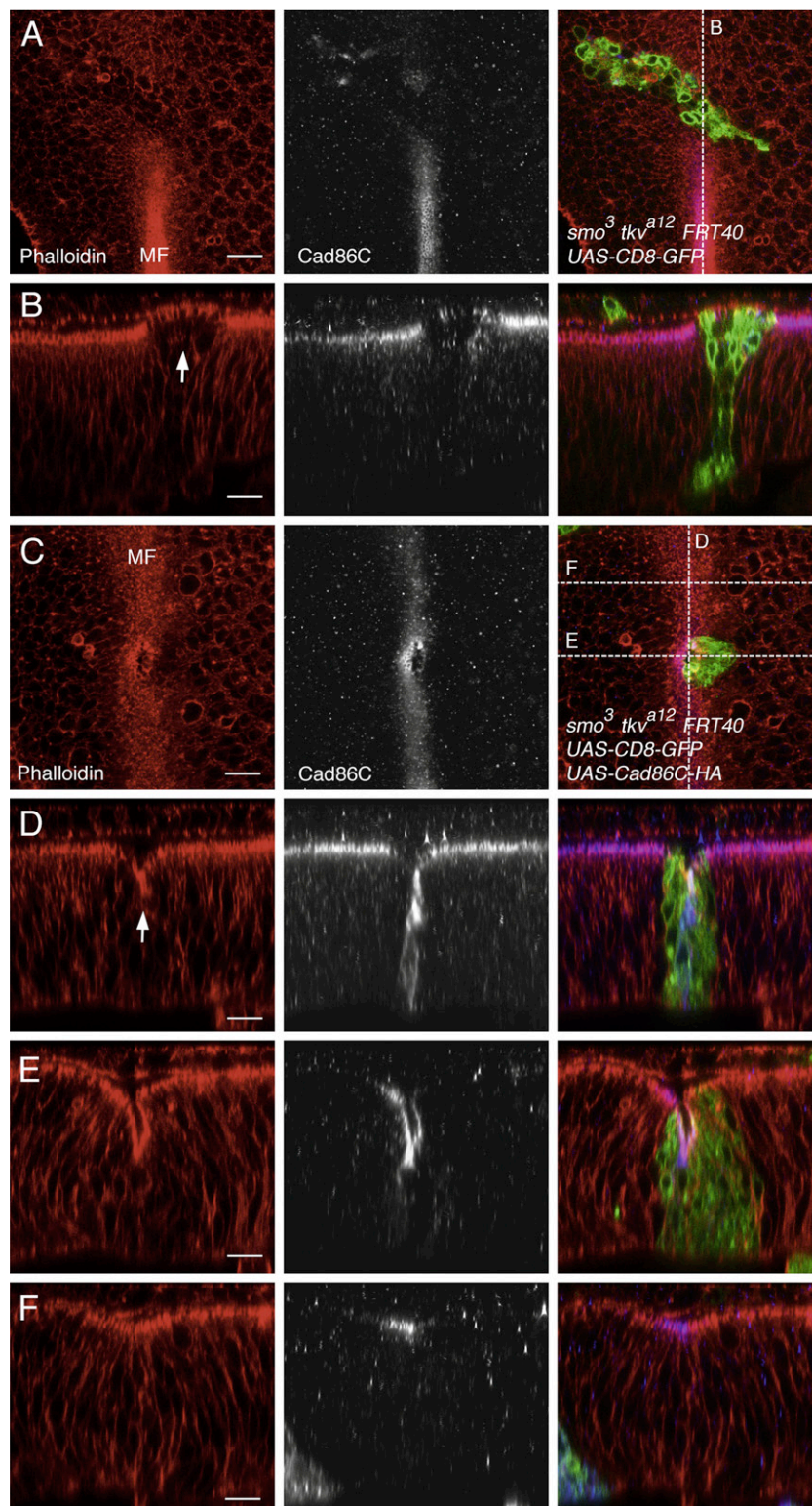
Myosin II has recently been shown to promote epithelial folding in the eye imaginal disc (Corrigall et al., 2007; Escudero et al., 2007). Our finding that Cad86C-EXTRA-HA promotes epithelial folding indicates that Cad86C does not directly interact with non-muscle Myosin II to bring about cell shape changes. Future studies will need to examine the relationship between Cad86C and non-muscle Myosin II.

3.3. Progression of the morphogenetic furrow

We found that both the Hedgehog and Dpp signaling pathways operate to promote the cell shape changes that normally occur in the morphogenetic furrow. While this manuscript was in preparation, two groups independently reported similar results (Corrigall et al., 2007; Escudero et al., 2007). It is tempting to speculate that Cad86C acts downstream of Hedgehog and Dpp signal transduction to promote the progression of the morphogenetic furrow. This speculation is mainly based on three observations. First, Cad86C protein is present at high levels in cells of the leading flank of the morphogenetic furrow, the cells that undergo apical constriction and shortening first. Second, Cad86C expression in the eye imaginal disc is regulated by Hedgehog and Dpp signal transduction, the two signal transduction pathways that promote the progression of the morphogenetic furrow. And third, ectopic expression of Cad86C-HA in clones of cells results in apical cell constriction and cell shortening, cell shape changes typically associated with the progression of the morphogenetic furrow. However, as we failed to detect a genetic requirement for Cad86C in morphogenetic furrow progression, it remains possible that Cad86C may play a role during eye development that is unrelated to morphogenetic furrow progression.

The morphogenetic furrow moves at a speed of one ommatidial cluster in approximately two hours. Based on our results and the results of others, we propose the following model of how the morphogenetic furrow progresses. Cells leaving the morphogenetic furrow start to differentiate and express Hedgehog. Hedgehog signals anteriorly to induce the expression of dpp in cells of the morphogenetic furrow. In response to Hedgehog and Dpp signaling, several target genes, including Cad86C, are induced in cells of the leading flank of the morphogenetic furrow. The resulting proteins promote the apical constriction and shortening of the leading edge cells, a process recently shown to require non-muscle Myosin II (Corrigall et al., 2007; Escudero et al., 2007). The apical constriction and shortening of the leading edge cells then moves the leading flank of the furrow anteriorly. As cells pro-

ceed through the center of the morphogenetic furrow, Hedgehog signal transduction is switched off and target gene expression ceases. Downregulation of Cad86C expression in the center of the morphogenetic furrow appears to be important, since sustained expression of Cad86C-HA prevents cells from elongating (Fig. 5A–C). The cessation of target gene expression, therefore, might allow cells to extend to their normal length and shape and, thus, to leave the morphogenetic furrow. These cells will then start to differentiate and express Hedgehog.

Cad86C possesses an unique activity to induce elongated folds in epithelia. The identification of Cad86C interacting proteins will be important to elucidate the mechanisms by which Cad86C promotes epithelial fold formation. Identification of Cad86C interacting proteins, as well as the identification of additional Hedgehog and Dpp target genes, promises to shed further light on the cell biological mechanisms underlying morphogenetic furrow progression.

4. Experimental procedures

4.1. Molecular cloning

The 5′ end of the Cad86C transcript was identified using a protocol described previously (Schlichting et al., 2005). The sequence of the gene specific primers were 5′-TTCTTCCGTTTTCG CCTTGTCCG-3′ and 5′-TGGGCTTCGTTCGGGTATCTGGAG-3′. The resulting PCR product was cloned using the TOPO TA Cloning kit (Invitrogen) and the inserts of four clones were sequenced. The sequence immediately 5′ of the predicted translational start codon matches the Drosophila translational start consensus sequence (AGAA) (Cavener, 1987).

The UAS-Cad86C-HA transgene was generated by first obtaining a cDNA including the entire coding sequence of Cad86C by RT-PCR using the primers 5′-AATGTGGACGCTGAACTGCTGAC G-3′ and 5′-TTG<u>GCGCGC</u>CGAGTACTGCGGCGACTCTCCG-3′ (the underlined sequence is a BssHII restriction site used for cloning). The cDNA was then cloned 5′ to, and in frame with, a triple HA epitope-encoding sequence derived from the influenza virus hemagglutinin protein HA1 (Wilson et al., 1984), and finally inserted into the pUAST vector (Brand and Perrimon, 1993). The UAS-Cad86C-EXTRA-HA transgene was generated by amplifying by PCR the coding sequence for amino acids 1–964 of Cad86C, comprising the extracellular and transmembrane regions, using the primers 5′-CGG<u>GAATTC</u>GGGTTGCATAAATCAGGCGAC-3′ and 5′-TTG<u>GCGCGC</u>TGGACACCAGGAGCAGATGCAG-3′ (the underlined sequences are EcoRI and BssHII restriction sites used for cloning). The PCR product was then cloned 5′ to, and in frame with, the triple HA-epitope-encoding sequence and inserted into the pUAST vector. For generating the UAS-Cad86C-INTRA-HA transgene, two different PCRs were performed. First, the coding sequence for

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Fig. 8 – **Expression of Cad86C-HA promotes shortening of Hedgehog and Dpp signaling compromised cells straddling the morphogenetic furrow. (A) Clones of cells homozygous mutant for smo^3 tkv^{a12} are marked by the presence of GFP staining (green). Rhodamine-phalloidin staining is shown in red and Cad86C is shown in white (blue in merge). (B) A cross section of the eye imaginal disc shown in (A). The position of the cross section is indicated by a dotted line in (A). Mutant cells fail to shorten (arrow). (C) Clones of cells homozygous mutant for smo^3 tkv^{a12} expressing Cad86C-HA are marked by the presence of GFP staining (green). Rhodamine-phalloidin staining is shown in red and Cad86C is shown in white (blue in merge). (D–F) Cross sections of the eye imaginal disc shown in (C). The positions of the cross sections are indicated by dotted lines in (C). smo^3 tkv^{a12} mutant cells expressing Cad86C-HA are short and form deep epithelial invaginations (arrow) compared to neighboring control cells within the morphogenetic furrow. Scale bars: 10 μm.**

amino acids 1–150 of Cad86C, containing the signal peptide (amino acids 119–137), was amplified using the primers 5′-CGGAATTCGGGTTGCATAAATCAGGCGAC-3′ and 5′-AACTGCAGGCGCATGCGCGTGGTCGGATC-3′ (the underlined sequences are EcoRI and PstI restriction sites used for cloning). Second, the coding sequence for amino acids 928–1943 of Cad86C, containing the transmembrane and cytoplasmic regions, was amplified using the primers 5′-CGGAATTCCTGCAGTACAAGGCCGAGAACAAG-3′ and 5′-TTGGCGCGCCGAGTACTGCGGCGACTCTCCG-3′ (the underlined sequences are PstI and BssHII restriction sites used for cloning). The two PCR products were then ligated, cloned 5′ to, and in frame with, the triple HA-epitope-encoding sequence, and inserted into the pUAST vector. For generating the UAS-E-cad-Cad86C transgene, the coding sequence for amino acids 1–1302 of Drosophila E-cadherin, containing the extracellular region, was amplified using the primers 5′-CGGAATTCGATCGCAGTTTCAAAATCGGTGTGG-3′ and 5′-CCAATGCATTCCCAAGTTGTAAGTCTGCTCG-3′ (the underlined sequences are EcoRI and NsiI restriction sites used for cloning) using UAS-CAD (Sanson et al., 1996) as template. The cloned PCR product was then ligated to the coding sequence for amino acids 928–1943 of Cad86C, containing the transmembrane and cytoplasmic regions. The resulting construct was then cloned 5′ to, and in frame with, the triple HA-epitope-encoding sequence, and inserted into the pUAST vector. For each of the transgenes, the correct nucleotide sequence of the cloned PCR products were confirmed by sequencing prior to injection into y w embryos to obtain transgenic flies.

4.2. Bioinformatic analysis

The domain organization of Cad86C was predicted using the Simple Modular Architecture Research Tool (SMART) (Schultz et al., 1998) and the putative signal peptide was identified using Signal IP (Nielsen et al., 1997).

4.3. RNA in situ hybridization

RNA in situ hybridization was performed as described previously (Schlichting et al., 2005). The Cad86C RNA probe corresponds to the coding sequence for amino acids 1442–1862.

4.4. Western blot of imaginal discs

Western blots of imaginal discs were performed as described previously (Schlichting et al., 2005). Primary antibodies used were rabbit anti-Cad86C (M0881; 1:10,000) and mouse anti-α-tubulin (1:10,000: Sigma T9026). Secondary antibodies used were goat anti-rabbit HRP-conjugated (1:10,000; Jackson Immuno Research 111-035-144) and goat anti-mouse HRP-conjugated (1:10,000; Pierce Prod. 31430).

4.5. Drosophila stocks

Mutant alleles for Cad86C were generated by imprecise excision of EP-element GE22560 (GenExel,Inc.). Out of 469 excision lines analyzed by PCR, two contained large deletions in the Cad86C gene. The allele $Cad86C^{184A}$ contains a genomic deletion of 13,571 bp spanning from 2735 bp 5′ to 10,833 bp 3′ of the translational start codon deleting the entire coding sequence. The allele $Cad86C^{71C}$ contains a genomic deletion of 10,361 bp spanning from 2735 bp 5′ to 7623 bp 3′ of the translational start codon deleting the coding sequence for amino acids 1–1001. The mutant allele of Cad88C is GE25091, an insertion of EP-element GE25091 into the coding region of Cad88C (GenExel,Inc.). Additional mutant alleles used were smo^3, a null allele of smo (Alcedo et al., 2000; Chen and Struhl,

1998), tkv^{a12}, a null allele of tkv (Nellen et al., 1994), hh^{ts2}, a temperature-sensitive allele of hh (Ma et al., 1993), and shg^{IH}, a loss-of-function allele of shg (Tepass et al., 1996). The following transgenic fly lines were used: Act5C>CD2>GAL4 (Pignoni and Zipursky, 1997), $UAS-ci^{PKA4}$ (Methot and Basler, 2000), $UAS-tkv^{Q253D}$ (Nellen et al., 1996), GMR-GAL4 (Freeman, 1996), UAS-CD8-GFP (Lee and Luo, 1999), and ap-GAL4 (Calleja et al., 1996). A y w stock was used as control.

4.6. Clonal analysis

Marked clones of mutant cells were generated by Flp-mediated mitotic recombination (Golic and Lindquist, 1989; Xu and Rubin, 1993), subjecting larvae to a 35–38 °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:

smo^3 FRT40: y w hsp-flp; smo^3 FRT40/ubi-GFP FRT40

tkv^{a12} FRT40: y w hsp-flp; tkv^{a12} FRT40/ubi-GFP FRT40

smo^3 tkv^{a12} FRT40: y w hsp-flp; smo^3 tkv^{a12} FRT40/ubi-GFP FRT40

Act5C>GAL4 $UAS-ci^{PKA4}$: y w hsp-flp; Act5C>CD2>GAL4/UAS-ci^{PKA4}

Act5C>GAL4 $UAS-tkv^{Q253D}$: y w hsp-flp; Act5C>CD2>GAL4/UAS-tkv^{Q253D}

FRT82 $Cad86C^{184A}$: y w hsp-flp; FRT82 $Cad86C^{184A}$/FRT82 hsp70-CD2

FRT82 $Cad86C^{71C}$: y w hsp-flp; FRT82 $Cad86C^{71C}$/FRT82 hsp70-CD2

Act5C>GAL4 UAS-Cad86C-HA: y w hsp-flp; Act5C>CD2>GAL4/UAS-Cad86C-HA

Act5C>GAL4 UAS-Cad86C-INTRA-HA: y w hsp-flp; Act5C>CD2>GAL4/UAS-Cad86C-INTRA-HA

Act5C>GAL4 UAS-Cad86C-EXTRA-HA: y w hsp-flp; Act5C>CD2>GAL4/UAS-Cad86C-EXTRA-HA

Act5C>GAL4 UAS-E-cad-Cad86C-HA: y w hsp-flp; Act5C>CD2>GAL4/UAS-E-cad-Cad86C-HA

smo^3 tkv^{a12} FRT40; UAS-CD8-GFP: y w hsp-flp; smo^3 tkv^{a12} FRT40A/tubP-GAL80 FRT40A; UAS-CD8-GFP

smo^3 tkv^{a12} FRT40; UAS-CD8-GFP UAS-Cad86C-HA: y w hsp-flp; smo^3 tkv^{a12} FRT40A/tubP-GAL80 FRT40A; UAS-CD8-GFP/UAS-Cad86C-HA

FRT42 shg^{IH}; $Cad86C^{71C}$/$Cad86C^{184A}$: y w hsp-flp; FRT42 shg^{IH} / FRT42 ubi-GFP; $Cad86C^{71C}$/$Cad86C^{184A}$

4.7. Immunohistochemistry

Imaginal discs dissected from late third instar larvae were fixed and stained according to standard protocols. The Cad86C antibodies M0881 and M0885 were generated by immunizing rabbits with GST–Cad86C fusion proteins containing amino acids 1069–1400 and 318–487 of Cad86C, respectively. The resulting sera were affinity purified and used at 1:10,000 dilution. Additional primary antibodies were mouse monoclonal anti-GFP, 1:100 (Santa Cruz Biotechnology), rabbit anti-GFP, 1:2000 (Clontech), rabbit anti-HA Y11, 1:1500 (Santa Cruz Biotechnology), mouse anti-DPatJ, 1:250 (Bhat et al., 1999), rat anti-DE-cadherin (DCAD2), 1:100 (Oda et al., 1994), rat anti-Ci 2A1, 1:4 (Motzny and Holmgren, 1995), rat anti-Elav, 1:5 (O'Neill et al., 1994), mouse anti-Armadillo N27A1, 1:100 (Developmental Studies Hybridoma Bank), and mouse anti-CD2 OX34, 1:2000 (Serotec). Secondary antibodies, all diluted 1:200, (Molecular Probes) were anti-mouse Alexa 488, anti-rabbit Alexa 488, anti-rabbit Alexa 594, anti-mouse CY5, anti-rat CY5. Rhodamine-phalloidin (Molecular Probes) was used at a 1:200 dilution. Images were recorded on a LSM510 Zeiss confocal microscope. The pixel intensity plot shown in Fig. 3E was generated using ImageJ. Background intensity was first subtracted and pixel intensity was then measured in a 6.7-μm wide stripe centering on

the peak intensity of Cad86C staining in the morphogenetic furrow. The GenBank Accession Number for the *Cad86C* sequence reported here is EU707853.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2008.04.005.

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