Phosphorylation Potential of Drosophila E-Cadherin Intracellular Domain is Essential for Development and Regulating Adherens Junction Biosynthetic Dynamics

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SUMMARY

E-Cadherin intracellular domain contains a highly conserved serine cluster whose phosphorylations are essential for binding to β-Catenin in vitro. In cultured cells phosphorylations of specific serine residues within the cluster are also required for regulating adherens junction (AJ) stability and dynamics. However, much less is known how such phosphorylations of E-Cadherin regulate the AJ formation and dynamics in vivo. In this report we generated an extensive array of Drosophila E-Cadherin (DE-Cad) endogenous knock-in alleles that carry mutations targeting this highly conserved serine cluster. Our mutant analyses suggest that the overall phosphorylation potential, other than the potential site-specific phosphorylations, of serine cluster enhance the recruitment of β-Catenin by DE-Cad in vivo. Moreover, phosphorylation potential of the serine cluster only moderately increases β-Catenin in AJ and is in fact dispensable for AJ formation in vivo. Nonetheless, phosphorylation-dependent recruitment of β-Catenin is essential for development, likely by enhancing the interactions between DE-Cad and α-Catenin. Specific phospho-mutants also dramatically affect the biosynthetic turn-over of mutant DE-Cad during apical-basal polarization and specifically rescued the polarity defects in embryonic epithelia lacking polarity proteins Stardust and Crumbs.
INTRODUCTION

Adherens junction (AJ) complexes are composed of transmembrane protein E-Cadherin and cytosolic proteins β-Catenin (β-Cat) and α-Catenin (α-Cat) (Harris and Tepass, 2010). Direct binding between β-Catenin and E-Cadherin intracellular tail is essential for AJ complex formation and trafficking. Such interaction also recruits α-Catenin that links AJ complex to the F-actin network (Buckley et al., 2014). Previous studies have identified a highly conserved serine cluster in E-Cadherin intracellular tail whose phosphorylations in vitro drastically increase E-Cadherin’s binding affinity to β-Catenin by ~800 fold (Choi et al., 2006; Huber and Weis, 2001; Lickert et al., 2000). In addition, cell culture studies showed that phosphorylations of specific serine residues within the cluster could play distinct roles in regulating AJ formation and stability (Choi et al., 2015; Lickert et al., 2000; McEwen et al., 2014). In *Drosophila*, we previously reported that the biosynthetic turn-over of AJs is differentially regulated during apical-basal polarization in *Drosophila* embryonic epithelia (Huang et al., 2011). The increased AJ stability in polarized cells coincides with the stronger binding between *Drosophila* E-Cadherin (DE-Cad, also known as Shotgun or Shg) and β-Catenin (also known as Armadillo or Arm (Peifer and Wieschaus, 1990)) (Huang et al., 2011), making the DE-Cad phosphorylations an attractive mechanism for modulating the DE-Cad/β-Catenin interaction during cell polarization.

Nonetheless, to date it remains to be fully characterized whether phosphorylations of the conserved serine cluster in E-Cadherin play significant roles in AJ formation, development and cell polarity in vivo. Recent studies in *C. elegans* based on transgenic expression of phospho-mutant E-Cadherins suggest a site-specific phosphorylation requirement in serine cluster for development, but it is unknown how β-Catenin binding is affected in vivo in such mutants (Choi et al., 2015). Using our genomic engineering method (Huang et al., 2009) we generated an extensive array of *Drosophila* DE-Cadherin knock-in alleles carrying specific deletions and phospho-mutations in the conserved serine cluster. One unique advantage of these knock-in mutants is that all mutant DE-Cad proteins are expressed from the endogenous locus to allow clean and consistent genetic assays. Using exclusively such engineered *DE-Cad* knock-in alleles, we first aimed to confirm whether the conserved serine cluster is essential for DE-Cad’s function in AJ formation and development. Second, we aimed to determine how the phosphorylation potential of the serine cluster is required for DE-Cad to interact with β-Catenin and α-Catenin in vivo. Finally, we also aimed to identify potential site-specific phosphorylations that may interact with apical polarity complex Stardust(Sdt)/Crumbs(Crb) to regulate the AJ formation and dynamics during apical-basal polarization.
RESULTS AND DISCUSSION

A short motif containing the conserved phosphorylatable serine cluster in DE-Cad intracellular domain is essential for AJ formation in vivo

The intracellular tail of E-Cadherin contains two functional domains: a juxtamembrane domain that regulates steady-state levels of AJ complex through interacting with p120-catenin, and a C-terminal motif that is required for binding β-Catenin (Fig. 1A,B) (Nagafuchi et al., 1994; Oda et al., 1993; Pacquelet et al., 2003; Stappert and Kemler, 1994). To further narrow down the minimal motif in DE-Cadherin required for recruiting β-Catenin to AJ in vivo, we generated three DE-Cad knock-in mutants carrying 83aa (DE-CadΔβ), 60aa (DE-CadΔβS) and an internal 22aa (DE-CadΔS) deletions that remove the conserved serine cluster (Fig. 1A,B). Similar to DE-Cadnull(shg2), all three mutants are embryonic lethal (Table 1) with a severe loss-of-cuticle phenotype (Fig. 1C) that indicates a strong disruption of epithelial polarity and integrity. In larval wing disc epithelia, mutant cells expressing DE-CadΔβS and DE-CadΔS fail to form discernable AJs which are labeled by junctional β-Catenin staining, instead show cytosolic GFP puncta devoid of β-Catenin (Fig. 1D and Fig. 2A, all DE-Cad mutants generated in this report are tagged with GFP at the C-terminus). The mutant clones are also of small and rounded shapes characteristic of DE-Cadnull mutant clones (Tepass et al., 1996). Such phenotypes suggest a complete loss of β-Catenin binding to DE-CadΔβS and DE-CadΔS and are consistent with the requirement of β-Catenin binding for E-Cadherin’s trafficking to plasma membrane (Chen et al., 1999). Loss of β-Catenin recruitment by DE-CadΔS is not simply due to the shortening of the DE-Cad intracellular tail, as a DE-CadΔS+LK knock-in allele with ΔS deletion replaced with a generic 22aa linker (Varnai et al., 2006) showed phenotypes identical to DE-CadΔS (Fig. 2A). For the sake of clarity we will hereafter refer to this 22aa motif as “S-motif” (Fig. 1A).

The conserved serine residues are quantitatively required for DE-Cad functions in development

To identify whether any of the conserved serines in S-motif are specifically required for β-Catenin recruitment, AJ formation and development, we generated an array of DE-Cad phospho-mutants (Table 1 and Fig. 1A) which will be referred hereafter collectively as DE-Cad*. Most of DE-Cad* mutants carrying various combinations of single or multiple non-phosphorylatable S->A and/or phosphomimetic S->D mutations within the 1457SLSSLAS serine cluster that is nearly 100% conserved among the S-motifs of Drosophila and mammalian E-Cadherins (Fig. 1A). Viability tests (Table 1) suggest that DE-Cad* mutants remain viable as long as two or more serine residues remain in the cluster. In addition, none
of the four serine residues appears to be specifically required in vivo for DE-Cad functions in development (Table 1). Furthermore, \textit{DE-CadS4D} in which all four serine residues mutated to phosphomimetic Asp is homozygous viable, suggesting that regulating the phosphorylation of SLSSLAS cluster is not essential for development. Our data are consistent with a model that the overall phosphorylation levels, rather than phosphorylations of any specific serine residues in S-motif, are critical for DE-Cad function in vivo.

A recent study identified that phosphorylation of a conserved serine residue upstream of the SLSSLAS cluster directly regulates the stability of vertebrate E-Cadherin (McEwen et al., 2014). We thus generated mutants targeting \textit{1454Ser} which is the equivalent residue in DE-Cad (Fig. 1A). \textit{DE-Cad} knock-in mutants carrying single mutation of \textit{1454Ser\textgreater{}Ala} or Asp (i.e. “\textit{A-SSSS}” or “\textit{D-SSSS}”, Table 1) are viable but such mutations are lethal when combined with S4D mutations (i.e. “\textit{A-DDDD}”or “\textit{D-DDDD}”, Table 1), suggesting that only in S4D mutant the potential phosphorylation of \textit{1454Ser} becomes essential for generating sufficient negative charges on S-motif for recruiting β-Catenin (see below). Overall, in contrast to the results in cultured cells and in \textit{C. elegans}, our data suggest that none of the conserved phospho-serines is specifically required for essential DE-Cad functions in vivo, instead these serine residues appear to be quantitatively required for viability and development.

\textbf{Loss of phosphorylation potential of S-motif moderately reduces β-Catenin levels in AJs in vivo}

We further focused on a subset of \textit{DE-Cad}\textsuperscript{*} mutants to investigate how phospho-mutations on S-motif affect AJ formation and β-Catenin/DE-Cad interactions in vivo. We used FRT-mediated mitotic recombination to simultaneously generate mitotic clones of both wild type \textit{DE-Cad::GFP} and mutant \textit{DE-Cad\textsuperscript{*}::GFP} in wing disc epithelia. Since both wild type and mutant DE-Cad proteins are tagged with GFP, their levels can be quantified in the same sample by immunostaining of anti-GFP antibody, while co-immunostaining of anti-β-Catenin antibody can be used to simultaneously quantify β-Catenin levels. In contrast to mutant clones of \textit{DE-Cad\textbeta{}S}, \textit{ΔS} and \textit{ΔS+LK} that show complete loss of AJs in larval disc epithelia, clones of all the examined \textit{DE-Cad}\textsuperscript{*} phospho-mutants form apparently normal AJs in larval wing disc epithelia judged by GFP and β-Catenin stainings (Fig. 2A). Moreover, although in vitro studies showed that phosphorylations of serine cluster on the S-motif increase β-Catenin binding to E-Cadherin intracellular tail by several hundred fold (Huber and Weis, 2001), none of the AJs formed by DE-Cad\textsuperscript{*} phospho-mutants show more than 60% reduction of β-Catenin (Fig. 2A,C, Table 1). Even in AJs formed by non-phosphorylatable \textit{DE-CadS4A} or \textit{DE-CadS5A}, β-Catenin levels are only reduced to ~56% and ~46% of wild type AJs, respectively (Fig. 2A,C). In AJ complex immunoprecipitated from zygotic \textit{DE-
CadS4A mutant embryos, β-Catenin is also only reduced approximately three-fold (Fig. 2D,D').

Why AJs formed by non-phosphorylatable DE-Cad mutants such as S4A and S5A still contain high levels of β-Catenin? We noticed that S-motif is also rich in acidic residues such as Asp and Glu (Fig. 1A). In particular, 1451 Asp and 1466 Asp residues in DE-Cad are in the same locations of 857 Ser and 872 Ser in human E-Cad therefore may play phosphomimetic functions. We mutated both to Asn in DE-Cad2DN mutant and found that β-Catenin level is also reduced in AJs (Table 1). Taken together, our data suggest that the in vivo recruitment of β-Catenin to AJs depends on the total negative charges on DE-Cad S-motif that include basal charges from acidic Asp and Glu residues and additional charges from the phosphorylations of the conserved serine cluster.

**Covalently linking α-Catenin to DE-Cad compensates the loss of phosphorylation-dependent β-Catenin in AJ formation and development.**

Although removing potential phosphorylations on the S-motif only moderately reduces the β-Catenin in AJs, DE-Cad* mutants that reduce the β-Catenin levels by more than 30% in mutant AJs are consistently lethal (Fig. 2C, Table 1). Live imaging showed that DE-CadS4A maternal mutant embryos develop normally until germband retraction after which the epidermis appear to breakdown during dorsal closure (Movie S1 and S2), suggesting that AJs formed by DE-CadS4A may not be robust enough to support tissue remodeling in late embryogenesis. A major function of β-Catenin is to recruit α-Catenin which attaches AJ complex to F-actin in a tension-dependent mechanism (Buckley et al., 2014), and covalently linking α-Catenin to E-Cadherin can at least partially compensate the loss of β-Catenin in AJ formation (Bianchini et al., 2015; Desai et al., 2013; Nagafuchi et al., 1994; Pacquelet et al., 2003; Sarpal et al., 2012). Indeed, fusion of α-Catenin completely rescues the developmental lethality of DE-CadS4A (Fig. 2B, C). Importantly, the rescue is not due to DE-CadS4A::α-Cat recruiting more β-Catenin, as AJs formed by DE-CadS4A::α-Cat show no obvious increase of β-Catenin compared to AJs of DE-CadS4A. Fusion of α-Catenin also rescued the AJ formation defects of DE-CadΔβS and DE-CadΔS, but both fusion mutants remain lethal (Table 1) with very low level of β-Catenin recruited to AJs (Fig. 1B, and Fig. 2B, C). Our data suggest that the major in vivo function of phosphorylation-dependent recruitment of β-Catenin by DE-Cad may be limited to enhancing the interaction between DE-Cad and α-Catenin, as loss of such β-Catenin can be fully compensated by fusion of α-Catenin to DE-Cad for AJ formation and development. However, phosphorylation-independent recruitment of β-Catenin by DE-Cad has an essential role in development and cannot be compensated by covalent fusion of α-Catenin to DE-Cad.
The conserved serine residues regulate the biosynthetic turn-over of DE-Cad during apical-basal polarization.

To investigate whether phosphorylations of S-motif could regulate AJs turn-over dynamics, we carried out whole-cell FRAP assays (Huang et al., 2011) to specifically measure the biosynthetic turn-over rates of selected DE-Cad* mutants in polarizing and polarized cells (Fig. 3A, S1A,B and Table 1). Stage 9-11 embryos were selected for assaying polarizing epithelial cells and stage 15 embryos for assaying polarized cells. Whole-cell FRAP assays were performed in lateral epidermis (Huang et al., 2011). Although all DE-Cad* show reduced levels of β-Catenin in AJs in vivo, overall it appears that loss of phosphorylation potential of SLSSLAS motif in DE-Cad does not significantly increases the biosynthetic instability of DE-Cad in AJs (Fig. 3A and Table 1). In contrast, fusion of α-Catenin to DE-CadS4A dramatically reduced the DE-CadS4A turn-over by five times, making DE-CadS4A::α-Cat the most biosynthetically stable mutants we have characterized so far (Fig. 3A). Such stabilizing effect is not due to α-Cat fusion to DE-Cad alone, since wild type DE-Cad::α-Cat only showed mildly reduced biosynthetic turn-over in polarizing cells (Fig. 3A). In addition, DE-CadASSA has significantly reduced biosynthetic turn-over in polarizing cells and increased turn-over in polarized cells – essentially a reversed differential regulation pattern of AJ dynamics compared to the wild type DE-Cad during cell polarization (Huang et al., 2011). Consistent with our whole-cell FRAP results, latrunculin treatment also showed that in DE-CadS4A::α-Cat and DE-CadASSA embryos the AJ-localized DE-Cad, and presumably AJs, are more resistant to the loss of F-actin (Fig. 3B). The viability of DE-CadASSA and DE-CadS4A::α-Cat mutants demonstrates that Drosophila can tolerate a surprisingly wide range of DE-Cad biosynthetic turn-over rates during cell polarization and development.

Biosynthetically stable DE-Cad mutants rescue the polarity defects in sdt and crb embryonic epithelial cells.

Sdt and Crb form an apical polarity complex essential for establishing apical-basal polarity in early embryonic epithelial cells, and their mutant embryos show identical defects in AJs and apical-basal polarity (Bachmann et al., 2001; Hong et al., 2001; Tepass and Knust, 1993). It has been proposed that Sdt/Crb complex is specifically required in polarizing or remodeling cells subject to fast turn-over of AJs (Campbell et al., 2009). To directly test this hypothesis, we combined sdt or crb with biosynthetically stable DE-CadASSA or DE-CadS4A::α-Cat mutants. In DE-Cad; crb and DE-Cad::a-Cat; crb mutant embryos, DE-Cad and DE-Cad::α-Cat, as well as apical polarity maker Baz are all severely disrupted at early stages (Fig. 3C). In contrast, mutant embryos of DE-CadASSA; crb, or sdt; DE-CadASSA or sdt; DE-CadS4A-α-Cat showed dramatic rescue of AJ formation and polarity by restoring the apical
localization of mutant DE-Cad and Baz (Fig. 3C). In addition, although Sdt and Crb are mutually dependent on each other for localizing to apical membrane (Hong et al., 2001), mutant embryos of sdtnull; DE-CadASSA and sdtnull; DE-CadS4A-α-Cat also restored Crb apical localization. As expected, the more stable DE-CadS4A::α-Cat yielded much stronger rescue of AJ formation and polarity defects than DE-CadASSA (Fig. 3C). Our data support that Sdt/Crb complex is specifically required in polarizing cells undergoing fast AJ turn-over, while in polarized cells biosynthetically stable AJs make Sdt/Cbr dispensable. Interestingly, DE-CadASSA or DE-CadS4A-α-Cat did not rescue Baz localization in amnioserosa cells in crb[KO] or sdt[KO] mutant embryos (data not shown), suggesting that Sdt/Crb complex acts through mechanisms independent of AJ stability to maintain the polarity in these actively constricting cells (David et al., 2010; Flores-Benitez and Knust, 2015; Harden et al., 2002).

In summary, we showed that the conserved serine cluster motif is structurally required for AJ formation in vivo. Although due to lack of proper antibodies it was not possible for us to determine the phosphorylation levels of serine cluster in wild type and mutant DE-Cad proteins, the extremely conserved nature of S-motif and the phenotypes of DE-Cad phospho-mutants support that phosphorylation of S-motif in DE-Cad is highly likely in vivo. However, our in vivo data showed that there is a surprisingly strong phosphorylation-independent recruitment of β-Catenin by DE-Cad that appears sufficient for AJ formation. While phosphorylation-dependent recruitment of β-Catenin in AJs is indeed required for development, its role appears to be limited to enhancing the interaction between α-Catenin and DE-Cad/β-Catenin complex. In contrast to transgenic rescue assays showed that 1457Ser-equivalent residue in worm E-Cadherin is essential for development, our Drosophila knock-in mutants showed that none of the conserved serine residues is specifically required for AJ formation and development in vivo. Mechanisms reconciling such differences remain unclear to us, but our data is consistent with a model that total negative charges on the conserved serine cluster enhance the potential binding between Drosophila DE-Cad and β-Catenin in AJs. However, site-specific phosphorylations of the serine cluster can dramatically regulate the biosynthetic stability of DE-Cad proteins in AJs, and it will be of great interest to further identify the specific serine residues and kinases that are involved in such regulations.
MATERIALS AND METHODS

Fly genetics: Generation of DE-Cad knock-in alleles were carried out by genomic engineering approach as described previously (Huang et al., 2009). DE-Cad mutants were recombined with FRT-G13 chromosome for clonal analysis. Mutants of crb\(^{KO}\) and sdt\(^{KO}\) were described previously (Huang et al., 2009). Additional materials and methods including full list of fly stocks and genotypes of samples presented in figure panels are provided in Supplementary Information.
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Competing Interests
The authors declare no competing or financial interests.

Author Contributions

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REFERENCES


Figure 1. The conserved serine cluster in DE-Cadherin is required for AJ formation and development.

(A) Sequence alignment of the phospho-serine clusters from Drosophila (accession#: GI 17136470), human (GI 6682963) and mouse (GI 6753374) E-Cadherin intracellular domains. Shadowed box highlights the S-motif. Equivalent phospho-serines in human E-Cadherins are numbered in parentheses (Pacquelet and Rorth, 2005).

(B) Schematic illustration of DE-Cad structure and deletion/α-Cat fusion knock-in mutants. EC: extracellular cadherin domain. TM: transmembrane domain. Yellow box: intracellular tail. Gray box: α-Catenin.

(C) Cuticles prepared from zygotic homozygous embryos of selected viable and lethal DE-Cad mutants. Phenotype classifications are according to Tepass et al (Tepass et al., 1996): Class I mutants show partial loss of cuticle while Class IV mutants show complete loss of cuticle.

(D) Mitotic clones generated in FRT-G13 DE-CadΔβS::GFP / FRT-G13 His2Av::mRFP larval wing disc by heat shock. DE-CadΔβS::GFP clones are labeled by the absence of nuclear RFP marker (from His2Av::mRFP), while twin wild type clones are identified by the loss of DE-CadΔβS::GFP. Mutant cells show no discernable AJs by either GFP or β-Catenin staining and DE-CadΔβS::GFP only form intracellular puncta. However, DE-CadΔβS::GFP mutant cells maintained apical-basal polarity as evidenced by normal staining pattern of Baz and aPKC.

Scale bars: 50μm in B and 5μm in D.
Figure 2. Phosphorylation potential of the conserved serine cluster on S-motif regulates AJ formation.

(A, B). Immunostaining of larval wing disc epithelial clones of selected DE-Cad knock-in mutants. All mutant clones were generated against DE-Cad::GFP and are marked by loss of nuclear RFP. Wild type twin clones are marked by increased RFP expression.

(C). Measured GFP (i.e. DE-Cad*:::GFP) and β-Catenin intensities and GFP/β-Catenin ratios in AJs in mutant clones. DE-CadΔβS(i.e. shg2) DE-CadΔβS and DE-CadΔS mutant cells do not form AJs therefore not are included in the chart. Names of lethal DE-Cad* mutants are in red.

(D) β-Catenin levels in AJ complexes immunoprecipitated from zygotic homozygous embryos of DE-Cad::GFP wild type (WT) or mutants as specified.

(D’) Quantified β-Catenin/DE-Cad*:::GFP ratios from D, based on three separate rounds of
immunoprecipitation results.

*: $P<0.05$; **: $P<0.005$ (two-tailed t-test).

Scale bars in A and B: 5μm.
Figure 3. Phospho-serines regulate the biosynthetic stability of DE-Cad in AJs.

(A) Whole-cell FRAP results of selected DE-Cad* mutants in polarizing and polarized embryonic epithelial cells. *: P<0.05; **: P<0.005 (two-tailed t-test).

(B) In early embryonic epithelia (stage 9-11), latrunculin treatment severely disrupts the zonular AJs formed by wild type (WT) DE-Cad::GFP and DE-Cad::α-Cat::GFP. AJs formed by DE-CadASSA and DE-CadS4A::α-Cat are much more resistant.

(C) Rescue of AJ and polarity defects in crb[KO] and sdt[KO] mutant embryonic epithelial cells by DE-CadASSA (“ASSA”) and DE-CadS4A-aCat (“S4A-a-Cat”). WT: DE-Cad::GFP. WT-α-
Cat: DE-Cad::α-Catenin::GFP. Embryos were between stages 11-12 of embryogenesis. Images were captured at lateral epidermis. Scale bars in E and F: 5μm.
Table 1. DE-Cad knock-in mutants.

All DE-Cad knock-in mutants are tagged with GFP at the C-terminus. Point mutations of phospho-serines in each allele are marked in an “S-SSSS” format abbreviating for Ser-1454, 1457, 1459, 1460, and 1463 in DE-Cad (see Fig. 1A). Each lethal allele was also confirmed by their lethality over the shg\textsuperscript{2} null allele, to exclude the possibility of background lethal mutations. Lethal phase of each allele was determined in zygotic homozygotes. DE-Cad\textsuperscript{*}::GFP and β-Catenin levels in mutant AJs were normalized against measurements in AJs formed by wild type DE-Cad::GFP in neighboring twin clones. In parentheses are the numbers of FRAP assays (one assay per embryo, 3\textsuperscript{rd} and 4\textsuperscript{th} columns) or clones quantitatively measured in immunostaining assays (the last three columns). Due to slow recovery of many whole-cell FRAP samples it is impractical to record FRAP long enough to calculate the t\textsubscript{1/2} and mobile/immobile fractions, therefore we calculated recovery rates as %/min linear rate based on the recovery within the first five to ten minutes (Huang et al, 2011). V: viable. “\": not done. Quantitative data are presented as mean±s.d.
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<td>ADDA</td>
<td>S-ADDA</td>
<td>Pupal</td>
<td>\</td>
<td>\</td>
<td>\</td>
<td>0.67 ± 0.21 (3)</td>
<td>0.45 ± 0.13 (3)</td>
<td>0.68 ± 0.04 (3)</td>
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<tr>
<td>ADDD</td>
<td>S-ADDD</td>
<td>V</td>
<td>0.84 ± 0.26 (3)</td>
<td>0.19 ± 0.11 (3)</td>
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<tr>
<td>S4A</td>
<td>S-AAAAA</td>
<td>Embryonic</td>
<td>1.75 ± 0.68 (4)</td>
<td>0.47 ± 0.19 (6)</td>
<td>1.01 ± 0.17 (3)</td>
<td>0.56 ± 0.15 (3)</td>
<td>0.57 ± 0.21 (3)</td>
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<tr>
<td>S4D</td>
<td>S-DDDDD</td>
<td>V</td>
<td>1.06 ± 0.08 (4)</td>
<td>0.18 ± 0.12 (5)</td>
<td>0.90 ± 0.17 (5)</td>
<td>0.79 ± 0.15 (5)</td>
<td>0.88 ± 0.12 (5)</td>
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<td>S5A</td>
<td>A-AAAAA</td>
<td>Embryonic</td>
<td>0.93 ± 0.55 (3)</td>
<td>0.40 ± 0.13 (6)</td>
<td>0.47 ± 0.11 (4)</td>
<td>0.46 ± 0.21 (4)</td>
<td>0.94 ± 0.32 (4)</td>
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<td>S5D</td>
<td>D-DDDDD</td>
<td>Embryonic</td>
<td>0.75 ± 0.31 (6)</td>
<td>0.50 ± 0.27 (3)</td>
<td>0.94 ± 0.21 (8)</td>
<td>0.68 ± 0.13 (8)</td>
<td>0.74 ± 0.18 (8)</td>
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<td>1D4A</td>
<td>D-AAAAA</td>
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<td>\</td>
<td>0.68 ± 0.33 (3)</td>
<td>0.44 ± 0.28 (3)</td>
<td>0.62 ± 0.10 (3)</td>
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<td>1A4D</td>
<td>A-DDDD</td>
<td>Pupal</td>
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<td>0.82 ± 0.09 (3)</td>
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<td>0.61 ± 0.03 (3)</td>
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<tr>
<td>ΔβS</td>
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<tr>
<td>ΔS</td>
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<td>WT-αCat</td>
<td>S-SSSS-αCat</td>
<td>V</td>
<td>1.29 ± 0.13 (3)</td>
<td>0.49 ± 0.07 (3)</td>
<td>1.10 ± 0.28 (3)</td>
<td>0.83 ± 0.17 (3)</td>
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<td>S4A-αCat</td>
<td>S-AAAA-αCat</td>
<td>V</td>
<td>0.35 ± 0.08 (3)</td>
<td>0.12 ± 0.05 (5)</td>
<td>0.86 ± 0.17 (5)</td>
<td>0.52 ± 0.16 (5)</td>
<td>0.59 ± 0.15 (5)</td>
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<tr>
<td>( \Delta \beta S - \alpha \text{Cat} )</td>
<td>( \Delta S - \alpha \text{Cat} )</td>
<td>0.93 ± 0.24 (5)</td>
<td>0.13 ± 0.04 (5)</td>
<td>0.15 ± 0.04 (5)</td>
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<td>WT-( \alpha \text{Cat}\Delta \text{ABD} )</td>
<td>Larval</td>
<td>0.98 ± 0.09 (3)</td>
<td>0.32 ± 0.02 (3)</td>
<td>0.33 ± 0.01 (3)</td>
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<td>WT-( \alpha \text{Cat}\Delta \text{CBD} )</td>
<td>Larval</td>
<td>( \Delta \beta - \alpha \text{Cat} )</td>
<td>Embryonic</td>
<td>( \Delta \beta )</td>
<td>( \Delta S - \alpha \text{Cat} )</td>
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<td>S4A-( \alpha \text{Cat}\Delta \text{CBD} )</td>
<td>Larval</td>
<td>( \Delta \beta )</td>
<td>( \Delta S - \alpha \text{Cat} )</td>
<td>( \Delta \beta - \alpha \text{Cat} )</td>
<td>( \Delta \beta - \alpha \text{Cat} )</td>
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<tr>
<td></td>
<td>S4A-( \alpha \text{Cat}\Delta \text{CBD} )</td>
<td>( \Delta \beta - \alpha \text{Cat} )</td>
<td>Embryonic</td>
<td>( \Delta \beta - \alpha \text{Cat} )</td>
<td>( \Delta \beta - \alpha \text{Cat} )</td>
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<tr>
<td>D1451N</td>
<td>V</td>
<td>1.07 ± 0.10 (7)</td>
<td>0.78 ± 0.08 (7)</td>
<td>0.73 ± 0.05 (7)</td>
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<tr>
<td>D1466N</td>
<td>Larval</td>
<td>1.00 ± 0.14 (7)</td>
<td>0.68 ± 0.11 (7)</td>
<td>0.68 ± 0.08 (7)</td>
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<tr>
<td>2DN</td>
<td>Larval</td>
<td>1.08 ± 0.11 (6)</td>
<td>0.72 ± 0.08 (6)</td>
<td>0.67 ± 0.10 (6)</td>
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SUPPLEMENTARY INFORMATION

SUPPLEMENTARY MATERIALS AND METHODS:

Fly Stocks: In addition to knock-in alleles listed in Table 1, the following stocks were used: w; His2Av::mRFP (“Histone::RFP”, BL#23651); hs-FLP w1118; adv1/CyO (BL#6); hs-FLP w1118; FRT-G13 His2Av::mRFP DE-Cad::GFP; ovo-FLP w (BL#8727); FRT-G13 ovoD1-18/Dp(?;2)bwD S1 wgSp-1 Ms(2)M1 bwD/Cyo (BL#2125); y1 w N1/FM7c, twi-Gal4 UAS-2xGFP (“twi-GFP” on X chromosome, BL#6873); w1118; In(2LR)Gla, wgGla-1/Cyo, twi-Gal4 UAS-2xGFP (“twi-GFP” on 2nd chromosome, BL#6662); w1118; DrMio/TM3, twi-Gal4 UAS-2xGFP (“twi-GFP” on 3rd chromosome, BL#6663); cn1 shg2 bw1 sp1/Cyo (BL#3085); y w sdtXP1 FRT-9-2/FM7c (Hong et al., 2001); y w; crb11e22 / TM3 Sb e (gift from Dr. Knust (Klebes and Knust, 2000)).

Generation of larval mitotic recombinant clones and germline clones: For generating larval disc clones, virgin females of hs-FLP w; FRT-G13 His2Av::RFP DE-Cad::GFP were crossed with males of y w/Y; FRT-G13 DE-Cad*. Parental flies were transferred every two days under 25°C. At 72 and 96 hours after egg laying vials containing the first and second instar larvae after the transfer were heat-shocked in a 37°C water bath for 1.5 hours per day. Five to six days after egg laying heat-shocked third instar larvae were dissected for wing discs which were fixed for 1 hour in 4% formaldehyde for immunostaining. Maternal and zygotic mutant embryos were generated by germline clones (GLC) according to the published protocol (Chou and Perrimon, 1996) except that ovo-FLP was used in lieu of hs-FLP.

Immunostaining and quantification: Immunostaining and quantification: Immunostaining of wind discs and embryos were described previously (Huang et al., 2009). Primary antibodies: chicken anti-GFP (Aves Lab, cat# GFP-1010) 1:5000; homemade rabbit anti-GFP (Huang et al., 2009) 1:1500; mouse anti-RFP (Thermo Fisher Scientific, MA5-15257) 1:500; mouse anti-β-catenin (DSHB, N2 7A1) 1:100; guinea pig anti-Baz (Huang et al., 2009); mouse anti-Crb (DSHB, cq4-c) 1:10; rabbit anti-aPKC (Santa Cruz, Sc-216) 1:1000. Secondary antibodies: Cy2-, Cy3 or Cy5-conjugated goat anti-rabbit IgG, anti-mouse IgG, and anti-guinea pig IgG (The Jackson ImmunoResearch Lab, 111-225-003, 115-165-003, and 106-175-003), all at 1:400. Images were collected on Olympus FV1000 confocal microscopes (Center for Biologic Imaging, University of Pittsburgh Medical School) and processed in Adobe Photoshop for compositions. The images containing Z-sections were analyzed by Image J and custom scripts in Photoshop. Junctional signals of GFP and β-Catenin were quantified by custom software as previously described (Huang et al., 2009).
Whole-cell FRAP assays and live imaging of embryogenesis: Staged the embryos were collected and their eggshells were manually removed. Dechorionated embryos were placed in air-permeable chambers filled with halocarbon oil (#95) on custom-made slides, to ensure their normal development throughout the imaging session (Huang et al., 2011). Whole-cell FRAP assays were carried out on a Nikon confocal microscope A1 (Center for Biologic Imaging, University of Pittsburgh, PA). Quantification of the FRAP recording images and data processing were described previously (Huang et al., 2011).

Cuticle preparation: Dechorionated embryos were mounted on slides in the mixture of lactic acid (S25374, Fisher Science Education) and Hoyer’s solution (1:1). The slides were baked under 65°C for 16 h before imaging. Lethal DE-Cad mutants were balanced on CyO, twi-GFP chromosome and zygotic mutant embryos were identified by the absence of GFP expression prior to cuticle preparation.

Immunoprecipitation of DE-Cad/β-Catenin complex from embryos: Embryos were collected for 2 hours at 25°C then aged for 4 hours at 25°C for collecting early embryos or 10 hours at 25°C for late embryos. The immunoprecipitation of AJ complex and western blot were carried out as previously described (Huang et al., 2009).

Drug treatments in embryos: Embryos were dechorionated in bleach, washed and then placed in 1:1 mixture of Schneider’s medium and n-octane (Teodoro and O’Farrell, 2003). Drugs or an equal amount of DMSO was added to the Schneider’s medium and embryos were shaken at 400 rpm for 30 minutes. Embryos were then quickly rinsed in n-octane, briefly dried in air and immediately mounted in halocarbon oil (#95) in an air-permeable chamber for imaging.

Genotypes of Drosophila Samples Presented in Figures:

Figure 1C:
“WT”: w^1118;
“S4D”: y w; FRT-G13 DE-CadS4D::GFP;
“S4A-αCat”: y w; FRT-G13 DE-CadS4A::α-Cat::GFP;
“ASSA”: y w; FRT-G13 DE-CadASSA::GFP;
“WT-αCat”: y w; FRT-G13 DE-Cad::α-Cat::GFP;
"ΔS-αCat": y w; FRT-G13 DE-CadΔS::α-Cat::GFP;
"S4A": y w; FRT-G13 DE-CadS4A::GFP;
"S5A": y w; FRT-G13 DE-CadS5A::GFP;
"ΔβS-αCat": y w; FRT-G13 DE-CadΔβS::α-Cat::GFP;
"KO": y w; FRT-G13 DE-CadKO::GFP;
"ΔβS": y w; FRT-G13 DE-CadΔβS::GFP;
"ΔS": y w; FRT-G13 DE-CadΔS::GFP;

Figure 1D:
hs-FLP w / y w (or Y); FRT-G13 DE-CadΔβS-αCat::GFP / FRT-G13 His2Av::RFP; +/-.
Cross: hs-FLP w; FRT-G13 His2Av::RFP / CyO (X) y w / Y; FRT-G13 DE-CadΔβS::α-Cat::GFP / CyO twi-GFP (X)

Figure 2A:
"WT": hs-FLP w / y w (or Y); FRT-G13 DE-Cad::GFP / FRT-G13 His2Av::RFP DE-Cad::GFP;
"ASSA": hs-FLP w / y w (or Y); FRT-G13 DE-CadASSA::GFP / FRT-G13 His2Av::RFP DE-Cad::GFP;
"S4D": hs-FLP w / y w (or Y); FRT-G13 DE-CadS4D::GFP / FRT-G13 His2Av::RFP DE-Cad::GFP;
"S4A": hs-FLP w / y w (or Y); FRT-G13 DE-CadS4A::GFP / FRT-G13 His2Av::RFP DE-Cad::GFP;
"KO": hs-FLP w / y w (or Y); FRT-G13 DE-CadKO::GFP/ FRT-G1 His2Av::RFP DE-Cad::GFP;
"ΔβS": hs-FLP w / y w (or Y); FRT-G13 DE-CadΔβS::GFP / FRT-G13 His2Av::RFP DE-Cad::GFP;
"ΔS": hs-FLP w / y w (or Y); FRT-G13 DE-CadΔS::GFP / FRT-G13 His2Av::RFP DE-Cad::GFP;
"ΔβS+LK": hs-FLP w / y w (or Y); FRT-G13 DE-CadΔβS+LK::α-Cat::GFP / FRT-G13 His2Av::RFP DE-Cad::GFP;
"2DN": hs-FLP w / y w (or Y); FRT-G13 DE-Cad2DN::GFP / FRT-G13 His2Av::RFP DE-Cad::GFP;
Figure 2B:

“WT-αCat”: hs-FLP w / y w (or Y); FRT-G13 DE-Cad::α-Cat::GFP / FRT-G13 His2Av::RFP DE-Cad::GFP;

“S4A-αCat”: hs-FLP w / y w (or Y); FRT-G13 DE-CadS4A::α-Cat::GFP / FRT-G13 His2Av::RFP DE-Cad::GFP;

“ΔβS-αCat”: hs-FLP w / y w (or Y); FRT-G13 DE-CadΔβS::α-Cat::GFP / FRT-G13 His2Av::RFP DE-Cad::GFP;

“ΔS-αCat”: hs-FLP w / y w (or Y); FRT-G13 DE-CadΔS::α-Cat::GFP / FRT-G13 His2Av::RFP DE-Cad::GFP;

Figure 2D: embryos were collected from the following stocks:

“WT”: y w; FRT-G13 DE-Cad::GFP

“ASSA”: y w; FRT-G13 DE-CadASSA::GFP

“S4A”: y w; FRT-G13 DE-CadS4A::GFP / CyO;

“SDDS”: y w; FRT-G13 DE-CadSDDS::GFP

“S4D”: y w; FRT-G13 DE-CadS4A::GFP

Figure 3A and Figure S1A,B:

“WT”: w^{1118};

“ASSA”: y w; FRT-G13 DE-CadASSA::GFP;

“S4D”: y w; FRT-G13 DE-CadS4D::GFP;

“S4A”: y w; FRT-G13 DE-CadS4A::GFPGLC ;(maternal and zygotic mutant embryo)

“WT-αCat”: y w; FRT-G13 DE-Cad::α-Cat::GFP;

“S4A-αCat”: y w; FRT-G13 DE-CadS4A::α-Cat::GFP;

Figure 3B:

“WT”: w^{1118};

“ASSA”: y w; FRT-G13 DE-CadASSA::GFP;

“WT-αCat”: y w; FRT-G13 DE-Cad::α-Cat::GFP;

“S4A-αCat”: y w; FRT-G13 DE-CadS4A::α-Cat::GFP;

Figure 3C:

“WT + crb^{K0}”: (stage 12 embryo)

y w; FRT-G13 DE-Cad::GFP; crb^{K0}

Stock: y w; FRT-G13 DE-Cad::GFP; crb^{K0}/TM3 twi-GFP
“ASSA + crb^{KO}”: (stage 10 embryo)
y w; FRT-G13  DE-CadASSA::GFP; crb^{KO}
Stock: y w; FRT-G13  DE-CadASSA::GFP; crb^{KO}/TM3 twi-GFP

“ASSA + sdt^{KO}”: (stage 11 embryo)
w sdt^{KO}/ Y; FRT-G13  DE-CadASSA::GFP
Stock: w sdt^{KO}/FM7c twi-GFP; DE-CadASSA::GFP

“WT-αCat + crb^{KO}”: (late stage 11 embryo)
y w; FRT-G13  DE-Cad-α-Cat::GFP; crb^{KO}
Stock: y w; FRT-G13  DE-Cad-α-Cat::GFP; crb^{KO} / TM3 twi-GFP

“S4A-αCat + sdt^{KO}”: (late stage 11 embryo)
w sdt^{KO}/ Y; FRT-G13  DE-CadS4A-α-Cat::GFP ;
Stock: w sdt^{KO} / FM7c twi-GFP; DE-Cad S4A-αCat::GFP
<table>
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<th>S4A-αCat</th>
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**Polarized Cells** (st.15 embryos)
Figure S1. Biosynthetic turnover of DE-Cad* mutants in embryonic epithelia measured by whole-cell FRAP assays.

(A-B) Representative whole-cell FRAP samples of selected DE-Cad* mutants in stage 9-11 early embryos (A, for measuring in polarizing embryonic epithelial cells) and stage 15 late embryos (B, for measuring in polarized embryonic epithelial cells). In each whole-cell FRAP sample, GFP in a patch of lateral embryonic epithelium was completely bleached therefore the GFP can only recover from de novo synthesis of DE-Cad. For presentation purpose images were processed in Photoshop to achieve enhanced contrast. All quantifications were done in original unadjusted images.
SUPPLEMENTARY MOVIES

Movie S1. Embryogenesis of maternal mutant embryo of *DE-CadS4A::GFP*. The movie is recorded by time-laps from starting stage 7 at 5min interval. The total recording time is 9 hours, with GFP channel at left and DIC channel at right. The genotype the embryo (*ovo-FLP w / +; FRT-G13 DE-CadS4A::GFP*) was confirmed by the absence of *twi-Gal4 UAS-GFP* expression. DE-CadS4A::GFP is too weak to be seen in this recording. Note the failed germ-band retraction starting 06:30 in DIC channel. Time stamp in “hh:mm” format.
Movie S2. Embryogenesis of zygotically rescued maternal mutant embryo of DE-CadS4A::GFP. The sample embryo was recorded simultaneously with the embryo in Movie S1. Wild type genotype (ovo-FLP w / +; FRT-G13 DE-CadS4A::GFP / CyO twi-GFP) was confirmed by twi-GFP expression starting 06:00. Time stamp in “hh:mm” format.