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Developing Strategies to Block Beta-Catenin Action in Signaling and Cell Adhesion During Carcinogenesis

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13. ABSTRACT (Maximum 200 Words)

To understand cancer, we must first understand normal cell behavior. *Drosophila* Armadillo (Arm) and its human homolog β-catenin are key players in adhesive junctions and in transduction of Wingless (Wg)/Wnt signals. Our working hypotheses are: 1) Several protein partners compete to bind Arm, and 2) Arm:dTCF activates Wg-responsive genes, while dTCF alone represses the same genes. Aim 1 is to understand how different partners compete with one another for binding Arm. Aim 2 focuses on how Arm and dTCF positively and negatively regulate Wg-responsive genes. In the past year we made significant progress. We used the two-hybrid system to further define the Arm binding site on DE-cadherin and extended our analysis of the effect of point mutations on binding. Our collaborators at the Weizmann Institute completed a parallel analysis in mammalian cells, assessing the ability of cadherin-derived peptides to compete β-catenin from its endogenous partners. This work was published in *Molecular Biology of the Cell*. We have also introduced into transgenic flies mutant versions of DE-cadherin which should specifically block Arm or p120catenin binding.

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Mark Green 6/6/01
PI - Signature Date
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To understand abnormal cell behavior in cancer, we must first understand normal cell behavior. We focus on Drosophila Armadillo (Arm); Arm and its human homolog β-catenin are critical for normal embryonic development (reviewed in Peifer, 1997). Both are key players in two separable biological processes: 1) They are components of cell-cell adhesive junctions, and 2) they act in transduction of Wingless/Wnt (Wg/Wnt) family cell-cell signals. Mutations in β-catenin or its regulators are early steps in colon cancer and melanoma (reviewed in Peifer and Polakis, 2000). We use the fruit fly as our model, combining classical and molecular genetics with cell biology and biochemistry. We take advantage of the speed and ease of the fly system and of its synergy with vertebrate cell biology. As one avenue to reveal Arm's roles in adherens junctions and transduction of Wg signal, we are identifying and examining the function of proteins with which Arm physically and/or functionally interacts. Our goal is to precisely define Arm/β-catenin's dual roles, ultimately allowing the design of drugs inhibiting oncogenic β-catenin. Our working hypotheses are: 1) Several protein partners compete to bind to the same site on Arm; the affinity of Arm for different partners is adjusted via phosphorylation of these partners, and 2) The Arm:dTCF complex activates Wg-responsive genes; dTCF represses the same genes in the absence of Arm. We will integrate approaches at all levels from combinatorial chemistry to studying gene function in intact animals, using fruit flies to carry out a functional genomics approach to understanding Arm function, and then transferring this knowledge directly to the mammalian system. Our first Aim is to understand how different partners interact with and compete with one another for binding Arm, and how phosphorylation regulates this. Our second Aim focuses on how the Arm and its partner dTCF positively and negatively regulate Wg responsive genes.

Specific Aim 1. Identify the sequence determinants mediating the binding of Armadillo/β-catenin's protein partners to Armadillo/β-catenin.

Specific Aim 2. Explore the mechanism of action of dTCF, a Wg/Wnt effector.

This award is a combined IDEA Award and Career Development Award. The IDEA component ends this year, while the CDA continues for another year. In the three years of the IDEA Award, we made significant progress on both of our Specific Aims, which we have outlined below.

Aim 1.
Our statement of work stated:
Year 1
1. Minimize interacting regions of all three partners and begin mutagenesis.
2. Carry out two-hybrid screen for random peptides that interact with Arm.
3. Mutagenize & test in two-hybrid system potential phosphorylation sites.
Year 2
5. Mutate potential phosphorylation sites in peptide models and test effects of GSK.
Year 3
6. Mutate regions required for Arm binding in the context of intact targets, reintroduce into flies and test for biological function.
7. Introduce peptides into cultured mammalian cells and test ability to bind β-catenin and block its function.

NOTE: Much of the work described in this section was recently published in a paper from our lab in Molecular Biology of the Cell (Simcha et al., 2001)—below we refer to Figures in this paper, which is included in the Appendix.

We previously found that dAPC, DE-cadherin, and dTCF all can bind to a ~260 amino acid fragment comprising Arm's Arm repeats 3-8 (Pai et al., 1996; van de Wetering et al., 1997) McCartney et al., 1999). In the first two years of work under this grant, we minimized the region of the DE-cadherin cytoplasmic tail required for Arm binding, defined a 22 amino acid region that was sufficient, using the yeast two-hybrid system as an assay (task 1 in the statement of work; Fig. 1 of Simcha et al., 2001) in Appendix). We also defined two different 34 amino acid portions of dAPC2, containing a single 15 amino acid repeat or a single 20 amino acid repeat, as sufficient for binding Arm (McCartney et al., 1999). Because such small regions were sufficient, we have not
pursued screening for random peptides that bound Arm (task 2).

We further extended these observations by beginning to examine the sequence requirements for Arm binding, beginning our examination by focusing on the DE-cadherin target. We based these experiments on both the enrichment of acidic amino acids in all of the targets of Arm, and on a slight but intriguing sequence similarity between Arm's partners. In particular, the motif SLSSL is conserved in APC and cadherin. This is of special interest because vertebrate E-cadherin and APC are phosphorylated in this region, most likely on these serines. In APC, phosphorylation of these serines by GSK-3 enhances β-catenin binding (Rubinfeld et al., 1996). In E-cadherin, serines in the region are phosphorylated by an unknown kinase; mutation of the serines to alanine blocks β-catenin binding (Stappert and Kemler, 1994). We thus made an extensive series of site-directed mutations of conserved residues (focusing in particular on acidic amino acids and on serines) within the minimal Arm binding region, including a small deletion and clustered point mutations (as outlined in tasks 1, 3, and 4 of the statement of work; Fig. 5 of Simcha et al., (2001) in Appendix). These tests were initiated in year two and completed in the last year. To our surprise, many of these mutations do not block binding to Arm when tested in the context of the full length cadherin tail. This suggests that multiple points of contact may underlie binding and that changes in individual contact sites may not be sufficient to block the interaction. However, the more extensive changes do abolish binding, and some of the lesser changes reduce binding detectably, beginning to reveal key residues (Fig. 5 of Simcha et al., (2001) in Appendix).

To supplement this work using the yeast two-hybrid system, we are carrying out a collaboration with Avri Ben'Zeev of the Weizmann Institute in Israel. We provided a series of mutant cadherin constructs which they then tested in cultured mammalian cells, using a series of assays which they have developed for examining the ability of the E-cadherin cytoplasmic tail to block β-catenin action (Sadot et al., 1998). They began this analysis in year two and have completed it in year 3. They have tested both our wild-type and mutant DE-cadherin constructs for their ability to bind to β-catenin in mammalian cells, when expressed as GFP-fusion proteins (tasks 4, 5, and 7). They examined the localization of these fusion proteins, their ability to block destruction of endogenous β-catenin (by competing for β-catenin binding with APC), their ability to block activation by the β-catenin-TCF/LEF complex (by competing for β-catenin binding with TCF/LEF), and their ability to block adherens junction formation (by competing for β-catenin binding with cadherin).

The results of these assays were quite revealing. First, we found that slightly longer portions of DE-cadherin are required in mammalian cells than are required in yeast, suggesting that competition with endogenous partners increases the stringency of the binding reaction Fig. 2-4 of Simcha et al., (2001) in Appendix). Second, we found that the effect of mutations was roughly parallel in yeast and in mammalian cells (with one exception), although all mutations tended to have a stronger effect in mammalian cells, likely due to competition with endogenous partners (Fig. 5-6 of Simcha et al., (2001) in Appendix). The exception was also quite revealing. While mutation of potential phosphorylation sites had little effect in yeast (Fig. 5 of Simcha et al., (2001) in Appendix), it resulted in striking reduction in binding in mammalian cells (Fig. 6 of Simcha et al., (2001) in Appendix), strongly supporting the idea that binding is normally regulated by phosphorylation (similar results were obtained by Lickert et al., (2000)). Finally, we found that different partners differ in their sensitivity to blockage by the cadherin peptide. The β-catenin-TCF/LEF interaction is most sensitive to disruption, while the β-catenin-E-cadherin interaction is least sensitive. The results of all of this work, both in yeast and in mammalian cells, were recently published in Molecular Biology of the Cell (Simcha et al., 2001), and the work from our lab in this paper was funded entirely by this IDEA Award. A copy is included in the Appendix, where the details of the methods used, the results, and their interpretation can be found.

While this manuscript was in preparation, a paper appeared that allowed us to carry our analysis to an even higher level. This paper reported the use of X-ray crystallography to solve the structure of a complex of Xenopus β-catenin and Tcf3 (Graham et al., 2000). This thus revealed in atomic resolution how β-catenin binds one of its partners. The authors also created a speculative model for how E-cadherin might bind β-catenin. Using our mutagenesis data, we evaluated and extended this model, creating a detailed prediction of how E-cadherin might interact with its partner (Fig. 7 of Simcha et al., (2001) in Appendix). This model was subsequently tested by the recent publication of the structure of complex of β-catenin and E-cadherin, also solved by X-ray crystallography (Huber and Weis, 2001). This will allow a further refinement of our understanding of how β-catenin binds diverse partners. Together, the crystal structure, our studies, and other mutagenesis studies of β-catenin's interaction with its partners (e.g., von Kries et al., 2000) will provide pharmaceutical companies with the insights they need to begin the rational design of inhibitors of interactions of β-
catenin and its partners, which might have therapeutic value in cancer or other diseases.

With this information in hand, we have now turned our attention using what we learned about the interaction of Arm and DE-cadherin to blocking particular interactions of E-cadherin and its catenin partners, and examining the consequences of this in vivo. Cadherins directly interact with at least two different cytoplasmic partners, Arm and the distantly related protein p120catenin. In work funded by other sources, we have employed a similar strategy to that described above to define the p120 catenin-binding site of the DE-cadherin tail (Lu et al., 1999). We found that fly p120catenin binds to the juxta-membrane region, similar to its mammalian partners (Thoreson et al., 2000; Yap et al., 1998). We then mutagenized the DE-cadherin tail and found clustered point mutations that block p120catenin binding in yeast. We have now introduced mutations that block Arm binding, defined by our work described above, and mutations that block p120catenin binding, into the intact DE-cadherin gene (Task 6 of the Statement of Work). Three of the four mutants, along with a wild-type control, have been introduced into flies. We are now beginning to test the effects of over-expressing these constructs in a wild-type background and asking whether they can rescue animals mutant for DE-cadherin, in collaboration with Ulrich Tepass of the University of Toronto.

Aim 2. Armadillo:dTCF, a bipartite transcription factor
Year 1
1. Construct, introduce into flies and begin to test effects of arm mutants with C-termini replaced with known activation and repression domains.
2. Examine genetic interactions between gro, wg, arm and dTCF mutations
Year 2
3. Complete analysis of arm-activation and repression domain fusions and initiate mutagenesis of Arm's C-terminus.
4. Examine physical interaction between Gro and dTCF in vitro and in vivo; Construct, introduce into flies and begin to test mutant forms of dTCF unable to bind Gro.
Year 3
5. Complete analysis of phenotypic consequences of mutations in Arm's C-terminus. Complete analyzing phenotype of mutant forms of dTCF unable to bind Gro.
6. Analyze the effects of mutant Arm constructs in cultured normal and transformed mammalian breast and colon epithelial cells.

In the three years of the IDEA Award, we made significant progress in our work on the role of Arm and dTCF in regulating Wg/Wnt responsive genes. As reported in the first annual report, in the first year, we explored in detail genetic and molecular interactions between gro, wg, arm and dTCF (task 2 and 4 above) – this work was a collaboration with the labs of Amy Bejsovec at Northwestern and Hans Clevers at Utrecht. These data, together with a parallel analysis of the interaction between Groucho homologs and TCF/LEF proteins in mammalian cells, demonstrated that TCF proteins play a dual role in the regulation of Wg/Wnt target genes. In the absence of Wg/Wnt signaling, they act together with the co-repressor Groucho to repress Wg/Wnt responsive genes. The rise in Arm levels triggered by Wingless signaling converts this repressor into an activator, leading to the expression of Wg-target genes. This work was published in Nature (Cavallo et al., 1998); reprint included in first year).

In year two, we explored in more detail the role of Arm's C-terminus in Wg signaling (Tasks 1, 3, and 5 of the Statement of Work). We found that C-terminally truncated mutant Armadillo has a deficit in Wg signaling activity, even when corrected for reduced protein levels. However, we also found that Armadillo proteins lacking all or part of the C-terminus retain some signaling ability if overexpressed, and that mutants lacking different portions of the C-terminal domain differ in their level of signaling ability. Finally, we found that the C-terminus plays a role in Armadillo protein stability in response to Wingless signal, and that the C-terminal domain can physically interact with the Arm repeat region. These data suggest that the C-terminal domain plays a complex role in Wingless signaling, and that Armadillo recruits the transcriptional machinery via multiple contact sites, which act in an additive fashion. These data were published in Genetics, with partial support from the IDEA Award (Cox et al., 1999); reprint included last year). While this work was underway, others examined the consequences of replacing the C-terminus of β-catenin with the Engrailed repressor domain (Tasks 1 and 3 of Statement of Work), and showed that, as we had hypothesized, this repressed Wnt target genes (Montross et al., 2000). Two other groups delineated activation domains in β-catenin and showed that a heterologous transactivator could mimic the properties of β-catenin’s C-terminus (Hsu et al., 1998; Vleminckx et al., 1999)
Career Development Award

The Career Development Award component of this grant pays a substantial portion of my salary (i.e., the PI, Mark Peifer). This has substantially reduced the amount of time I have to devote to teaching and service, and has thus allowed me to focus on research, both that funded by the Army and other research ongoing in my lab. I have thus acknowledged this support in additional publications produced during this period, which are listed in Section 8, and included reprints (where available) in the Appendix.

(7) Key research accomplishments for the entire 3 years.

a) A 22 amino acid piece of DE-cadherin is sufficient for Armadillo binding in the yeast two-hybrid system.

b) Clusters of 3-4 point mutations in conserved sequence motifs in DE-cadherin do not block Armadillo binding in the two hybrid system, while a subset of more extensive amino acid substitutions in this region do so.

c) The minimal cadherin peptides can compete for interaction with β-catenin in vivo, displacing its endogenous partners.

d) The effect of mutations in the core binding region parallels that assessed in yeast, with one exception.

e) In general, the requirements for interaction with β-catenin were more stringent in mammalian cells, where endogenous partners are present, than in yeast, where they are absent, suggesting that competition between partners likely regulates complex formation in vivo.

f) Mutation of known phosphorylation sites in De-cadherin dramatically reduces binding in mammalian cells, while it had little effect on binding in yeast, suggesting that phosphorylation regulates binding in vivo.

g) Our mutagenesis studies, combined with the crystal structure of the β-catenin:TCF complex, allowed us to make a prediction of the structure of the β-catenin:E-cadherin complex.

h) Binding to TCF/LEF is more easily competed than that to APC/Axin, and both are more easily competed than binding to E-cadherin.

i) Groucho binds dTCF

j) Groucho acts as a dTCF co-repressor in vivo, repressing Wingless-responsive genes and thus shaping pattern of the embryonic segment.

k) Armadillo's C-terminus plays multiple roles in Armadillo function.

(8) Reportable outcomes for the last year.

New publications in the last year supported in part by the IDEA grant:


New publications in the last year acknowledging partial salary support for Mark Peifer via the CDA:


Publications from the previous year acknowledging partial salary support for Mark Peifer via the CDA for which reprints are now included:


Degrees supported by this grant

1. Ph.D. awarded to Dr. Robert Cavallo, December 1999, entitled “New partners for Armadillo in signal transduction and cell adhesion”. A portion of the work in this thesis was supported by the IDEA Award.

2. M.S. awarded to Mr. Gordon Polevoy, April 2001, entitled “Mechanisms of Armadillo’s roles in signaling and adhesion.” Virtually all of the work in this thesis was supported by the IDEA Award.

Presentations by Mark Peifer discussing this work.


"Cell adhesion, signal transduction, and cancer: the Armadillo Connection”. Laboratory of Molecular Carcinogenesis, NIEHS/NIH, RTP NC October, 2000

"Cell adhesion, signal transduction, and cancer: the Armadillo Connection”. Memorial Sloan-Kettering Cancer Center, New York NY December, 2000

"Cell adhesion, signal transduction, and cancer: the Armadillo Connection”. Department of Molecular Biology, Princeton University, Princeton NJ February 2001

"Cell adhesion, signal transduction, and cancer: the Armadillo Connection”. Division of Biology, University of California at San Diego, La Jolla, CA, May 2001

(9) Conclusions.

We made significant progress on each of the specific aims. In carrying out Aim 1, we examined in detail the binding of DE-cadherin to Arm/β-catenin, identifying a very small region that is sufficient for binding in the yeast two-hybrid system and identifying within that region the key amino acids required for binding. We completed an analysis of binding of wild-type and mutant peptides in mammalian cells, in collaboration with our colleagues at the Weizmann Institute. This work was published in Molecular Biology of the Cell. These data should provide a basis for understanding the interaction between the protein product of the oncogene β-catenin and its mammalian partners in both normal development and physiology, and during oncogenesis.

In carrying out Aim 2, we found that dTCF plays a dual role in Wingless/Wnt signaling. We had previously shown that it acts together with Arm to activate Wingless target genes. In work funded in part by this grant, we found that in the absence of Arm, dTCF forms a complex with the co-repressor Groucho, and together they repress Wingless target genes. This work was published in Nature. We also examined the role of the C-terminus of Arm, which we had hypothesized acted as a transcriptional activation domain. We found that it plays a more complex role in Arm function. The C-terminus can be divided into two regions, which contribute in an additive way to Arm’s role as an activator of Wingless signaling. It also appears to regulate the stability of Arm. This work was published in Genetics. Subsequent work by others has confirmed the relevance of our results for mammalian β-catenin. Understanding the mechanism by which Wnt target genes are repressed will provide insight into the normal and abnormal regulation of the genes responsible for oncogenesis in tumors resulting from activation of the Wnt pathway.

(10) References.


Appendix — Reprints of publications supported in part by this grant

Publications supported in part by the IDEA grant:

Publications acknowledging partial salary support for Mark Peifer via the CDA:

Publications from the previous year acknowledging partial salary support for Mark Peifer via the CDA for which reprints are now included:

Cadherin Sequences That Inhibit β-Catenin Signaling: A Study in Yeast and Mammalian Cells

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Submitted November 6, 2000; Revised January 19, 2001; Accepted January 30, 2001
Monitoring Editor: Joan S. Brugge

**Drosophila** Armadillo and its mammalian homologue β-catenin are scaffolding proteins involved in the assembly of multiprotein complexes with diverse biological roles. They mediate adherens junction assembly, thus determining tissue architecture, and also transduce Wnt/Wingless intercellular signals, which regulate embryonic cell fates and, if inappropriately activated, contribute to tumorigenesis. To learn more about Armadillo/β-catenin's scaffolding function, we examined in detail its interaction with one of its protein targets, cadherin. We utilized two assay systems: the yeast two-hybrid system to study cadherin binding in the absence of Armadillo/β-catenin's other protein partners, and mammalian cells where interactions were assessed in their presence. We found that segments of the cadherin cytoplasmic tail as small as 23 amino acids bind Armadillo or β-catenin in yeast, whereas a slightly longer region is required for binding in mammalian cells. We used mutagenesis to identify critical amino acids required for cadherin interaction with Armadillo/β-catenin. Expression of such short cadherin sequences in mammalian cells did not affect adherens junctions but effectively inhibited β-catenin-mediated signaling. This suggests that the interaction between β-catenin and T cell factor family transcription factors is a sensitive target for disruption, making the use of analogues of these cadherin derivatives a potentially useful means to suppress tumor progression.

INTRODUCTION

Vertebrate β-catenin and its **Drosophila** homologue Armadillo (Arm) play critical roles in both cell adhesion and signal transduction (reviewed by Gumbiner, 1996; Willert and Nusse, 1998). These proteins are key effectors of Wingless (Wg)/Wnt signal transduction, interacting with DNA-binding proteins of the TCF/LEF family to form bipartite transcription factors that activate Wnt responsive genes (reviewed by Wodarz and Nusse, 1998). β-Catenin and Arm are also core components of the cadherin-catenin complex, which mediates cell-cell adhesion at adherens junctions and connects these junctions to the actin cytoskeleton (reviewed by Ben-Ze'ev and Geiger, 1998; Provost and Rimm, 1999). These quite distinct biological functions of β-catenin/Arm most probably rest on a similar biochemical role: β-catenin/Arm mediates assembly of multiprotein complexes. Thus, in adherens junctions, it simultaneously binds cadherins and α-catenin, whereas in the nucleus it links TCF/LEF proteins to the basal transcriptional machinery (reviewed by Zhurinsky et al., 2000a).

In addition to these roles in normal development and physiology, β-catenin is also a critical target in the development of a variety of human tumors (reviewed by Peifer and Polakis, 2000). In normal cells, β-catenin/Arm's role in signal transduction is kept off by targeting the protein for rapid proteolytic destruction. β-Catenin/Arm is targeted for destruction by a multiprotein complex, which includes two scaffolding proteins, APC and axin/conductin, and a kinase, GSK3β. Assembly of this complex leads to phosphorylation of β-catenin/Arm, and its subsequent ubiquitination and destruction. If this complex is disrupted by mutations in either APC (reviewed by Peifer and Polakis, 2000) or axin/conductin (Liu et al., 2000; Satoh et al., 2000) the Wnt pathway is activated. This can lead to cell proliferation and tumor initiation. Finally, β-catenin binds to a diverse set of other proteins, including the presenilins, the epidermal growth factor (EGF) receptor, the actin-binding protein fas-
had a modest effect on binding, whereas mutation of all eight conserved serines abolished binding in vivo. These data suggest a testable model for the interaction between \( \beta \)-catenin/Arm and its partners, in which charge-based and other interactions mediate the binding between the Arm repeats of \( \beta \)-catenin/Arm and short regions of its partner proteins, potentially binding as extended peptides in the basic Arm repeat groove. Here, we test this model for the interaction between \( \beta \)-catenin/Arm and its partners, by carrying out a detailed analysis of the sequence requirements for interaction between caderhins and \( \beta \)-catenin/Arm.

**MATERIALS AND METHODS**

**Cadherin Constructs and Yeast Two-Hybrid Experiments**

The Arm R1-12 construct in pCK2 was described by Pai et al. (1996); it was previously called Arm R1-13, but the subsequent crystal structure of \( \beta \)-catenin led to reassessment of repeat number and boundaries. Similar constructs containing Arm repeats 2-10 (ArmR2-10: amino acids 177-596) and the corresponding fragments of mouse \( \beta \)-catenin (R1-12: amino acids 119-708; R2-10: amino acids 169-583) were generated for this work. DE-cadherin fragments were generated by polymerase chain reaction (PCR) with flanking BamHI and EcoRI restriction sites and cloned into pCK4 (Pai et al., 1996). The amino acids included in each fragment are diagrammed in Figure 1, A and B. All constructs included a stop codon after the final amino acid of DE-cadherin. All clones were sequenced in their entirety to confirm their sequence. The DE-cadherin mutants (DEC) were generated by a two-step PCR procedure. Primers for each strand containing the desired mutant sequence were used in two separate PCR reactions with flanking primers to amplify the N- and C-terminal portions of the DE-cadherin cytoplasmic domain. Products from these two reactions were mixed and used as a template for another PCR reaction containing only the flanking primers. This reaction generated a full-length DE-cadherin cytoplasmic domain with flanking BamHI and EcoRI sites containing the desired mutations, which was cloned into pCK4. Mutation DEC2 was introduced into the smaller DEC30 fragment by amplifying the relevant portion of the longer mutant clone with DEC30 primers. All mutations were confirmed by sequencing and are diagrammed in Figure 6A. Two-hybrid assays were performed as described by Pai et al. (1996). Arm or \( \beta \)-catenin fragments were fused to the LexA DNA-binding domain in pCK2, and DE-cadherin fragments were fused to the Gal4 activation domain in pCK4. The two plasmids were transformed simultaneously into the yeast strain L40. \( \beta \)-Galactosidase values are the averages from duplicate assays performed on at least three independent transformants.

**Cell Lines and Transfections**

Chinese hamster ovary (CHO), 293T and MDCK cells were maintained in DMEM with 10% calf serum. Transient transfections with *Drosophila* E-cadherin (DEC) constructs were carried out using the calcium phosphate precipitation method with 293T cells and by Lipofectamine (GIBCO, Grand Island, NY) with CHO cells. For recloning the various mutant DEC sequences from the pCK4 plasmid, the DEC inserts were amplified by PCR using primers designed to contain pCK4 plasmid sequences (in the HA-tag domain) that were linked to the multicloning site, ACCTAGTCTTATGATACGATGTC- CAG, and the terminator sequence, CAGATAGATGAAGT- GGATCC, downstream of the multicloning site of pCK4. The amplified sequences were excised by BamHI and EcoRI digestion and inserted into pEGFP-C1 at the same BglII/EcoRI sites. The green fluorescence protein (GFP) tag was localized at the N terminus of

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**REFERENCES**

Conti, 1994; Lickert 1994; Stappert and Kemler, 1994; Lickert
residues that are phosphorylated in vivo (Stappert and Kem- inserted into pEGFP-C1 at the same
nin-binding site in E-cadherin focused on a series of serine amplified sequences were excised by

binding or contribute to it. Mutational analysis of the P3-cate- GAACTTGC, downstream of the multicloning site of pCK4. The

identifying critical amino acids that are either required for to the multicloning site, ACCTAGATCTTACCCATACGATGTTC-

levels, suggesting reduced binding to P-catenin (Rubinfeld mid into the pEGFP-C1 plasmid (Clontech, Palo Alto, CA), the DEC

sequence were used in two separate PCR reactions with flanking

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**REFERENCES**

Conti, 1994; Lickert 1994; Stappert and Kemler, 1994; Lickert
residues that are phosphorylated in vivo (Stappert and Kemler, 2000). Mutation of individual serines phosphorylated in vivo (Stappert and Kemler, 1994; Lickert et al., 2000). Mutation of individual serines
Figure 1. Mapping the minimal binding site on DEC cytoplasmic tail for β-catenin (βcat) and Arm using the yeast two-hybrid (2 hyb) system. (A) Schematic representation of the DEC derivatives used in our analyses, with ability to bind Arm/β-catenin in either yeast or mammalian cells summarized in the right-hand columns. TM, transmembrane; TC, tissue culture. *Data from Pai et al. (1996). (B) Sequence of the minimal binding region of DE-cadherin, with the boundaries of the smallest DEC derivatives indicated. (C) All of the DEC derivatives bind to both fragments of Arm and β-catenin in yeast. The full-length DE-cadherin cytoplasmic domain (DEC), or smaller derivatives of DEC (diagrammed in A and B), fused to the Gal4 transcriptional activation domain, were transformed into yeast cells along with portions of Arm or β-catenin fused to the LexA DNA-binding domain. Average β-galactosidase values are shown for each DEC derivative together with the full Arm repeat region of Arm or β-catenin (Arm R1-12 or βcat R1-12, left), or a smaller fragment of the Arm repeat region (Arm R2-10 or βcat R2-10, right). 0, background level of β-galactosidase activity with no DEC fragment fused to Gal4. **DEC 25 was tested against only Arm R1-12. Its β-galactosidase value was 14.4 U, compared with 18.3 U for the negative control, Boehringer Mannheim, Indianapolis, IN), polyclonal anti-HA (a gift from M. Oren, Weizmann Institute of Science, Rehovot, Israel), polyclonal anti-β-catenin (Sigma, St. Louis, MO), and monoclonal anti-GFP antibody (Roche Molecular Biochemicals, Burlington, NC). For coimmunoprecipitation, cells transfected with the GFP-DEC constructs and the S33Y β-catenin were lysed in immunoprecipitation buffer containing 20 mM Tris-HCl, pH 8.0, 1% Triton X-100, 140 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM MgCl₂, 1 mM dithiothreitol, 1 mM sodium orthovanadate, and 50 μg/ml phenylmethylsulfonyl fluoride. Equal amounts of protein were incubated with 2 μl of polyclonal anti-β-catenin antibody and 20 μl of protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h at 4°C. The beads were washed five times with 20 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl and 0.5% Nonidet P-40, and the immune complexes were recovered by boiling in Laemmli’s sample buffer and resolved by SDS-PAGE. To detect the coprecipitated GFP-DEC constructs, the blots were incubated with anti-GFP antibody. Blots were developed using the ECL method (Amersham,
**RESULTS**

**Mapping the Minimal Arm/β-Catenin-interacting Region of DE-Cadherin**

The binding site for Arm on the DE-cadherin cytoplasmic tail was previously mapped to the C-terminal portion of the cadherin tail (Pai *et al.*, 1996). To determine the minimum region essential for binding, we first used the yeast two-hybrid system to assess binding between the Arm repeat region of both Arm and β-catenin and smaller fragments of the DE-cadherin tail (Figure 1). A series of fragments, ranging in size from 23–34 amino acids, were tested, and all bound both Arm and β-catenin as assessed by the two-hybrid system (Figure 1C).

We then examined whether these minimal binding fragments, when fused to GFP at their N termini, retained the ability to interact with β-catenin in mammalian cells. To do so, we made use of several assays. First, we assessed the ability of the GFP-DE-cadherin tail and its fragments to coimmunoprecipitate with β-catenin (Figure 2A). Second, we tested the ability of these fragments to block the interaction of β-catenin with endogenous LEF/TCF, as measured by their ability to block LEF/TCF-mediated transactivation (Figure 2B). Finally, we assessed the capacity of these fragments to block the interaction of β-catenin with endogenous APC or axin, thus stabilizing β-catenin by blocking its targeting to the proteasome (Figure 2C).

**Defining Amino Acids Critical for the Arm/β-Catenin-DE-Cadherin Interaction**

We next set out to determine which amino acids within the minimal DE-cadherin–binding region were essential for the interaction with Arm and β-catenin. Mammalian β-catenin can bind DE-cadherin both in *Drosophila* (White *et al.*, 1998; Cox *et al.*, 1999) and in cultured mammalian cells (see below), and Arm can also bind mammalian E-cadherin (A. Wodarz and R. Nusse, personal communication). We therefore used the comparison of vertebrate and *Drosophila* cadherins to determine candidate residues that might contribute to binding. Based on comparisons of the Arm-binding regions of cadherin, APC, and TCF family members, we focused on acidic and serine/threonine residues, although we also mutated other conserved amino acids. Although we focused on residues within the minimal binding region, we introduced our mutations in the context of the full-length DE-cadherin tail, thus mimicking the situation in vivo.

We began with three clustered point mutations that each change three or four nearby residues in different regions of the minimal binding site to alanine (Figure 5A). DECM1 altered four conserved serine residues in the center of the minimal binding region, DECM2 altered four conserved amino acids including one acidic residue in the N-terminal part of the minimal binding region, and DECM3 altered three conserved acidic residues (aspartates) in the C-terminal part of the minimal binding region (Figure 5A). Surprisingly, none of these mutations significantly affected binding to the full-length Arm repeat region of either Arm or β-cate-
Figure 2. Analysis of the ability of different fragments of the DEC cytoplasmic tail to interact with β-catenin, affect its stability, and inhibit β-catenin-mediated transactivation.

(A) The ability of selected GFP-DEC derivatives to coimmunoprecipitate with cotransfected HA-tagged β-catenin was determined by immunoprecipitation (IP) from 293T cells transfected with HA-tagged β-catenin and GFP-tagged DEC constructs with anti-GFP antibody, followed by Western blotting with anti-HA antibody. The total level of transfected β-catenin and DEC constructs was determined by immunoblotting (IB) with anti-HA antibody. (B) 293T cells were transfected with GFP-tagged derivatives of the DEC cytoplasmic tail (DEC) or the full-length mammalian E-cadherin tail (E), along with β-catenin (β), a LEF/TCF reporter plasmid (T), and Lac Z. Luciferase activity was determined from duplicate plates as fold activation after normalizing for transfection efficiency by measuring β-galactosidase activity. T, cells were transfected with the reporter plasmid alone; V, cells transfected with the reporter plasmid, HA-tagged β-catenin and the GFP-vector used for the construction of the cadherin derivatives. (C) The cadherin derivatives used in B were transfected into CHO cells, and their ability to protect the endogenous β-catenin from degradation was determined by analyzing the level of β-catenin expressed in the DEC mutant-transfected cells by Western blotting with anti-β-catenin antibody. The level of expression of DEC constructs was determined by immunoblotting with an antibody against the GFP tag. Quantitation of the β-catenin level expressed in CHO cells was carried out by normalizing the intensity of the β-catenin bands shown to those of the DEC band for each derivative.
Figure 3. The effect of DEC cytoplasmic domain derivatives on the organization of adherens junctions and subcellular distribution of β-catenin (β-cat). MDCK cells were transfected with various GFP-tagged DEC derivatives (diagrammed in Figure 1, A and B), and the distribution of the GFP-tagged DEC derivatives (A, C, E, G, and I) and of the endogenous β-catenin (B, D, F, H, and J) was determined by double fluorescence microscopy using rhodamine-labeled anti-β-catenin antibody. Bar (in A), 10 μm. Note the reduction in junctional β-catenin in DEC-expressing cells but not in cells transfected with other DEC constructs. Also note that DEC9 and DEC29 do not increase the endogenous β-catenin level, whereas DEC13 does.

Figure 4. Analysis of the ability of DEC derivatives to increase the level and the accumulation of endogenous β-catenin (β-cat) in the nucleus. Some of the GFP-DEC constructs described in Figure 3 were transfected into CHO cells (A, C, and E), and their ability to elevate the endogenous β-catenin and induce its translocation into the nucleus (B, D, and F) was determined by double fluorescence as described in Figure 3. Bar in (A), 10 μm. The arrows mark the transfected cells. Note that, whereas DEC13 and DEC induced the accumulation of endogenous β-catenin in the nucleus, DEC9 was unable to do so.

changes. We therefore tested the DECM1-DECM3 mutants for their capacity to bind to Arm repeats 2-10 of both Arm and β-catenin (Figure 5B, right). In this assay, there was a substantial reduction in the binding of DECM2 to both Arm and β-catenin, whereas the other two mutations (DECM1 and DECM3) did not substantially affect binding (Figure 5B, right). These data suggested that DECM2 might weaken binding but not enough to be detectable in the context of the full-length DE-cadherin tail binding to the full-length Arm repeat region. Interactions outside the minimal Arm-binding region may normally help stabilize this association and thus could partially compensate for mutations such as DECM2. We therefore introduced the DECM2 mutation into a 34-amino acid peptide centered on the minimal binding region.
Figure 5. Analysis of the effect of clustered point mutations in the minimal Arm-binding domain of DEC on its ability to bind Arm and β-catenin (βcat) in the yeast two-hybrid system. (A) Diagram of the DEC tail and sequences of the clustered point mutations used in this study, with the sequences of DE-cadherin (DE-Cad) and human E-cadherin (hE-Cad) in the region of the mutations shown below. All mutations were introduced into and analyzed in the context of the full-length cytoplasmic tail. The mutation DECM2 was also tested in the context of a smaller fragment of the cadherin tail (DEC30; Figure 1A)—this derivative is DECM3(M2). (B) The DE-cadherin mutants diagrammed in A were fused to the Gal4 transcription activation domain and transformed into yeast cells together with the full Arm repeat region of Arm or β-catenin (Arm R1-12 or βcat R1-12, left) or a smaller fragment of the Arm repeat region (Arm R2-10 or βcat R2-10, right), fused to the LexA DNA-binding domain. Average β-galactosidase activities are shown.

(DEC30; Figure 1A). In this context (rather than in the full-length DEC tail), the DECM2 mutation essentially abolished binding to even the full-length Arm repeat region (DEC30(M2); Figure 5B).

Next, we tested this same set of mutants for binding to β-catenin in cultured mammalian cells, using the assays described above. Both DECM1 and DECM3 retained substantial ability to block TCF-directed gene expression (Figure 6A), suggesting that they could block binding of β-catenin to TCF family members. All three mutants were reduced in their ability to stabilize β-catenin (Figure 6B), although all appear to retain a small amount of activity in this assay. Finally, the overexpression of DECM3 in MDCK cells (Figure 6C) resulted in the accumulation of β-catenin in the cytoplasm and nuclei of these cells (Figure 6D).

In addition to these mutations, we also analyzed three mutants with more substantial changes in the minimal binding region. Mutant DECM7 combined the changes found in DECM1 and DECM2 and also mutated an additional amino acid, aspartic acid 1450, to valine (Figure 5A). Mutant DECM8 altered all of the serine and threonine residues in the core of the Arm repeat site to alanine (Figure 5A) and also altered the semiconserved residue glycine 1455 to aspartic acid. A subset of these residues is a likely target of phosphorylation in vivo (Stappert and Kemler, 1994). Finally, in DECM10, 20 amino acids were deleted in the core of the minimal binding region (Figure 5A). When tested against the full Arm repeat region of Arm or β-catenin in the two-hybrid system, DECM7 and DECM10 were essentially inactive (Figure 5B). In contrast, DECM8 had little effect on binding to the entire Arm repeat region (Figure 5B, left), although it did reduce binding to Arm repeats 2–10 of both Arm and β-catenin (Figure 5B, right). We also analyzed an additional mutant, DECM9, in which four of the conserved serine residues were changed to glutamic acid (Figure 5A). These serine residues are phosphorylated in vivo, and in some cases, this change mimics phosphorylation. DECM9 retained full ability to bind both Arm and β-catenin in the two-hybrid assays (Figure 5B).

DISCUSSION

β-Catenin/Arm plays key roles in cell-cell adhesion and Wnt signal transduction. Deregulation of these activities...
Figure 6. Analysis of the effect of clustered point mutations in the minimal Arm-binding domain of DEC on its capacity to interact with β-catenin, protect it from degradation, inhibit β-catenin/LEF-mediated transactivation, and affect β-catenin organization. (A) The ability of clustered point mutations (diagrammed in Figure 5A) to affect β-catenin/LEF-1-mediated transactivation in 293T cells was examined as described in Figure 2B. (B) The ability to protect β-catenin from degradation was examined in CHO cells, and the levels of β-catenin were quantified as described in Figure 2C. Because the samples were originally analyzed on the same gel with the samples shown in Figure 2C, the control samples (V, DEC, and D9) are shown again. (C–F) MDCK cells were transfected with GFP-tagged DECM3 (C, M3) and DECM8 (E, M8), and the organization of the endogenous β-catenin (β-cat) in the respective samples (D and F) was determined by double fluorescence microscopy. Bar, 10 μm.
can lead to disease. Activation of β-catenin–mediated signaling contributes to a wide variety of human tumors (reviewed by Zhurinsky et al., 2000a), and dysfunction of cadherin-catenin adhesion is involved in cancer metastasis (reviewed by Christofori and Semb, 1999). β-Catenin/Arm mediates these distinct processes by forming a scaffold upon which different multiprotein complexes are assembled. To unravel β-catenin's normal functions and the alterations in its function in disease, a detailed understanding of its interactions with protein partners is required. This might facilitate a rational approach to design inhibitors of these interactions. For example, an effective, specific inhibitor of the β-catenin–TCF interaction might have therapeutic potential in cancers in which Wnt signaling is activated.

We used the cadherin/β-catenin interaction as a model for investigating this question. We previously found that 71 amino acid derivatives of the cytoplasmic tail of vertebrate N- or E-cadherin inhibit β-catenin/TCF-mediated transactivation when introduced into human colon cancer cells (Sadot et al., 1998; Simcha et al., 1998). Moreover, expression of the N-cadherin tail in human colon cancer cells inhibited the elevated transcription of cyclin D1 (Shutman et al., 1999), thus potentially suppressing its oncogenic function. In the present study, we analyzed the interaction between DE-cadherin and β-catenin/Arm in detail, using several assays, each of which provided different measures of binding. Using the yeast two-hybrid system, we assessed interaction in the absence of most, but not all, of β-catenin/Arm's normal partners, because yeast lack β-catenin, cadherins, TCFs, APC, and axin. Furthermore, kinases and other proteins that regulate interactions between β-catenin/Arm and its partners, are also likely absent. We also used several assays in mammalian cells, which, in contrast to yeast, possess both a full (or nearly full) complement of β-catenin partners and the normal set of regulatory machinery that modulates the interaction between β-catenin and its partners. This diversity of assays allowed us to discriminate among the binding abilities of cadherin mutants in a more detailed way than was possible in most previous studies of β-catenin/Arm interaction with other partners, which, for the most part, relied on single assays.

Using these assays, we found that quite small fragments of DE-cadherin, including the 23-amino acid DEC27, bind both β-catenin and Arm in yeast. In cultured mammalian cells the criteria for interaction were more stringent. The smallest DE-cadherin peptide that interacted in mammalian cells was DEC28, which is 27 amino acids in length. This difference may reflect the fact that in mammalian cells DE fragments must compete with endogenous partners for binding—weakened interactions might prevent effective competition. Alternately, it may simply reflect differences in the fusion proteins used in each assay. It is noteworthy, however, that the binding of short DE fragments, such as DEC28 in mammalian cells, is weaker than binding of the full cytoplasmic tail of DE-cadherin or mammalian E-cadherin, as assessed by their ability to inhibit transcriptional activation by β-catenin (Figure 2B).

Our mutational analysis also revealed critical amino acids in cadherin required for interaction with β-catenin. The β-catenin/Arm-binding site is highly conserved among all classical cadherins. Most of our mutations in conserved residues had parallel effects in yeast and mammalian cells. For example, mutation of three acidic amino acids near the C terminus of the minimal binding region (DEC3M3) had little effect on either binding in yeast or the ability to block TCF-mediated transactivation, whereas mutation of four more N-terminal conserved residues (DEC2M2) resulted in a detectable reduction in binding in yeast and a substantial reduction in the ability to block TCF-mediated transactivation. The most extensive mutations, DEC7M and DEC10, completely blocked the binding in all assays.

Surprisingly, the serine residues in the binding site, mutated in DEC1M and DEC8M, were largely dispensable for binding in yeast. In contrast, these mutations impaired or eliminated the ability to block TCF-mediated transactivation and to stabilize β-catenin in mammalian cells. One possible explanation for these differential effects is that these serines are phosphorylated in mammalian cells (Stappert and Kemler, 1994; Lickert et al., 2000); this may strengthen binding. Consistent with this possibility, mutation of the four conserved serines to glutamic acid (mutant DEC9M), which may mimic phosphorylation, did not block binding to β-catenin in mammalian cells. In fact, DEC9M very effectively protected β-catenin against degradation (Figure 2C), in agreement with recent studies by Lickert et al. (2000). If, in yeast, the relevant kinase(s) are absent, mutation of these serines would not affect binding.

While this paper was under review, two studies appeared that complement our data. Graham et al. (2000) solved the structure of β-catenin bound to XTcf3, thus revealing in full detail how β-catenin binds to one of its partners. XTcf3 binds in the groove on the surface of β-catenin, with the XTcf3 peptide forming a β-hairpin at its N terminus and an α-helix at its C terminus, with an extended peptide in between. From this structure and parallel mutagenesis of β-catenin, they identified two key charge-charge interactions between β-catenin and the extended XTcf3 peptide and a key hydrophobic interaction of β-catenin with the α-helix of XTcf3. They also assessed the ability of cadherin to bind to their β-catenin mutants and, from this, proposed a model for how cadherins bind β-catenin.

Based on our data, we extended this model, as shown in Figure 7A. In addition to the sequence similarity noted by Graham et al. (2000) in the extended peptide region, we suggest a further sequence alignment in the α-helical region. Notably, the three XTcf3 residues, which they identified as critical for interaction with β-catenin, are conserved in diverse cadherins (boxed in Figure 7A). Although the spacing between the extended peptide and the α-helix differs between TCF and cadherins, this region of XTcf3 is disordered in the structure and may form a flexible loop, and if fully extended, the cadherin peptide could span the gap. We also noted a similar, although less striking, alignment of the 20 amino acid repeats of APC and XTcf3, with all three key residues also conserved (Figure 7B).

Our mutational analysis can also be examined in light of this structure (Figure 7A). Mutation DEC2M2, which has a severe effect on binding, alters four amino acids including a glutamic acid predicted by analogy to XTcf3 to mediate
and all are conserved in all sequences. The amino acids altered in mutation DEC7 (YEG G), which have a strong effect on DEC binding to β-catenin/Arm in our assays, are bold and underlined. The amino acids altered in mutation DEC8 (DD D), which had the weakest effect on binding, are shown in italics. The serines altered in mutations DEC1 and DEC9 are in italics and underlined. B) Alignment of the XTcf3 sequence and structure with four 20-amino acid repeats of Drosophila APC (dAPC).

one of the key charge-charge interactions with β-catenin (Figure 7A, bold underline). In contrast, mutation DEC3, which had no effect in yeast and the least severe effect in mammalian cell assays, maps to a region predicted by analogy to be outside the structured portion of the binding site (Figure 7A, italics). The analysis of mutations DEC1 and DEC9 is more complex. Mutation of the four serines targeted in DEC1 to alanine has no effect in yeast but substantially reduces binding in mammalian cells. In contrast, mutation DEC9, which altered these serines to glutamic acid, did not affect binding. Of these four serines, the second and third align with serines in XTcf3. The second serine is predicted to be on the face of the α-helix away from β-catenin, whereas the third serine does not contribute to binding. The first serine is a valine in XTcf3, which participates in hydrophobic contacts, whereas the fourth serine is predicted by analogy to XTcf3 to be beyond the end of the α-helix and to have its side chain pointed away from β-catenin. If, as discussed above, these serines are phosphorylated, then the first and third phosphoserines might make charge-charge interactions with lysine 292 of β-catenin; this would also be the case if they were mutated to glutamic acid.

von Kries et al. (2000) also revealed new insights into β-catenin's interaction with its partners. They mutagenized β-catenin to identify amino acids in the Arm repeat region, which are essential for binding to APC, axin/conductin, and TCF/LEF. They found that mutations mapping to distinct Arm repeats blocked binding to individual partners. Thus, LEF-1 binding was inhibited by mutations in Arm repeat 8, whereas conductin binding was inhibited by mutations in Arm repeats 3 and 4. These data suggest that either different partners bind to distinct sites on β-catenin or, if the binding sites coincide, different subsets of the contacts between β-catenin and each of its partners provide most of the free energy of binding. In parallel, they also examined whether these β-catenin mutations affected binding to E-cadherin (J.P. von Kries and W. Birchmeier, personal communication). In contrast to their results with the other partners, none of the mutations specifically blocked β-catenin binding to cadherin. Graham et al. (2000) also tested mutant forms of β-catenin for binding to XTcf3, C-cadherin, APC, and axin. XTcf3 binding required two key charge-charge interactions with the extended peptide region and a key hydrophobic interaction with the α-helix. For cadherin, mutations predicted from the structure to block the key charge-charge interactions reduced binding, but mutations in the α-helix–binding region had little effect. These data are of interest in relation to the present study in which, contrary to expectations, none of the first series of clustered point mutations (DEC1, DEC2, and DEC3) abolished DEC binding to β-catenin in yeast. One possible explanation for all these results is that the binding of cadherins to β-catenin differs from that of the other partners, with strong contacts made throughout the binding region. Thus, point mutations in either cadherin or β-catenin would have a lesser effect on binding. This might also explain the apparently higher affinity of cadherin for Arm in vivo, as assessed by competition for the limiting pool of Arm present in arm mutant embryos (Cox et al., 1996).

We assessed our mutations in the full DE-cadherin cytoplasmic tail. We also assessed DEC7 in a second context, introduced into a 34-amino acid fragment centered on the minimal binding region. In this context, DEC7 had a much more severe effect on β-catenin binding in yeast than it did when present in the full DE-cadherin tail. This result is consistent with the possibility that β-catenin binding is stabilized by interactions with regions of the cadherin tail outside the minimal binding domain or that the entire tail folds into a conformation that facilitates β-catenin binding.
To affect one function of β-catenin without affecting the others, one must design inhibitors that specifically interfere with a particular protein–protein interaction. Our results provide some insight into this issue. Both wild-type and mutant cadherin peptides were more effective in blocking interaction of endogenous β-catenin with TCF/LEF than in blocking interactions between endogenous β-catenin and the axin/APC complex or assembly of adherens junctions. This would be the desired outcome for a specific inhibitor that blocked the oncogenic action of β-catenin. Our data also suggest possible peptide candidates for cocrystallization of cadherin and β-catenin. When combined with the β-catenin–TCF structure, this would set the stage for initiating the design of synthetic inhibitors of different protein–protein interactions, which can be tested in cell culture and animal models for efficacy in blocking Wnt signaling or modulating cell adhesion and cancer progression.

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REFERENCES


Cadherins not only maintain the structural integrity of cells and tissues but also control a wide array of cellular behaviours. They are instrumental for cell and tissue polarization, and they regulate cell movements such as cell sorting, cell migration and cell rearrangements. Cadherins may also contribute to neurite outgrowth and pathfinding, and to synaptic specificity and modulation in the central nervous system.

In 1963, two publications appeared that focused attention on the adhesive mechanisms that contribute to embryonic morphogenesis and govern structural differentiation of the nervous system. Malcolm Steinberg laid the groundwork for the 'differential adhesion hypothesis', suggesting that the segregation or 'sorting out' of different embryonic cell types into separate tissues involves qualitative or quantitative differences in cell adhesion. Roger Sperry proposed the 'chemoaffinity hypothesis', which holds that specific synaptic contacts form according to differences in the adhesive properties of individual growth cones and synapses. Both hypotheses were the result of decades of experimental work, but were formulated before any adhesion molecules had been identified. It is now apparent that a large number of adhesion molecules exist that can be grouped into several superfamilies. The cadherin and immunoglobulin-type adhesion molecules are the main groups of cell–cell adhesion receptors, whereas the integrins are the predominant contributors to cell–substrate adhesion. Adhesive mechanisms that contribute to embryonic or neural morphogenesis share many similarities, revealing that Steinberg's and Sperry's hypotheses are essentially similar proposals applied to different cell populations.

Morphogenesis involves two interrelated themes: structure and movement. For example, the different adhesive properties of two mixed cell populations induce cell movement, leading to the sorting out of the two groups of cells. After the sorting process is completed, adhesive differences maintain the segregation and relative position of the two cell groups, therefore preserving a specific tissue architecture. Neuronal morphogenesis follows a similar pattern, in which the neuronal growth cone has to move through a complex environment using differential adhesive cues. On reaching the target, synaptic contacts are formed and maintained by specific adhesive interactions.

The first cadherins to draw scientists' attention were vertebrate classic cadherins, which were independently identified for their ability to mediate calcium-dependent adhesion among cultured cells and for their role in the epithelialization of the early mouse embryo. So far, the sequences of over 300 vertebrate cadherins have been reported, and the virtually complete sets of cadherins encoded by the genomes of Caenorhabditis elegans and Drosophila melanogaster are now known.

Structural diversity of the cadherin superfamily

The recent explosion in genomic sequencing of various animals has shed new light on the diversity of the cadherin superfamily. In humans, more than 80 members of the cadherin superfamily have been sequenced. Current annotation of the C. elegans and the Drosophila genomes reveals 14 and 16 cadherin genes, respectively. Cadherins are defined by the presence of the cadherin domain (CD), a roughly 110 amino-acid peptide that mediates calcium-dependent homophilic interactions between cadherin molecules (FIG. 1). The CD is typically organized in tandem repeats. Calcium ions associate with the linker region that connects two CDs, and require interaction with amino acids from both CDs (FIG. 1; see below).

Here we present a classification of cadherins into
subfamilies on the basis of the domain layout of individual cadherins, which includes the number and sequence of CDs, and the presence of other conserved domains and sequence motifs (FIG. 2, TABLE 1). This analysis reveals that four cadherin subfamilies are conserved between C. elegans, Drosophila and humans: classic cadherins, Fat-like cadherins, seven-transmembrane cadherins and a new subfamily of cadherins that is related to Drosophila Cad102E. Classic cadherins break up in four subgroups, as listed in Table 1. Fat-like cadherins contain a subgroup of highly related molecules that we call Fatoid cadherins. These include all known vertebrate Fat-like cadherins, Drosophila Cad76E and C. elegans Cdh-4. Cadherins containing protein kinase domains are found in vertebrates (RET cadherins) and in Drosophila. Desmosomal cadherins are presumably derived from type I classic cadherins within the chordate lineage, as neither desmosomal cadherins nor desmosomes are found in Drosophila or C. elegans. Finally, protocadherins also seem to be limited to chordates. The grouping of cadherins into seven subfamilies, which is largely on the basis of the overall protein domain architecture, is corroborated by sequence comparison of CDs only (see online supplementary materials). Note that only about half of the cadherins found in C. elegans and Drosophila are part of identified subfamilies.

Cadherins are not found in yeast, and only a single, poorly conserved CD has been reported in a secreted protein from Dictyostelium, indicating that transmembrane proteins of the cadherin superfamily might have evolved to meet the need for the complex cell interac-

tions that are required for the multicellular organization of metazoans. The function of classic cadherins during the formation and maintenance of epithelial tissues and cell-cell adhesion functions — two other metazoan inventions — is of particular importance. Within the classic cadherin subfamily, an interesting shift in protein organization has taken place during evolution — chordate classic cadherins lack non-chordate classic cadherin domains (NCCDs), laminin G (LG) and epidermal growth factor (EGF) domains and consistently contain five CDs, in contrast to the domain structure of classic cadherins in three other phyla: echinoderms, arthropods and nematodes (FIG. 2, TABLE 1). This finding indicates that, during the early evolution of chordates, the structure of classic cadherins was modified and that a single progenitor might have given rise to the numerous classic cadherins found in vertebrates today, a conclusion supported by the phylogenetic analysis of chordate cadherins.

The cadherin families of C. elegans and Drosophila are small and very similar in size, and these cadherin genes are scattered throughout the genome without any obvious clustering, indicating that gene duplication events might not have occurred recently. So most of these genes were presumably established early during metazoan evolution. In particular, the progenitors of the four conserved subfamilies of cadherins are the result of a gene radiation event that occurred before nematodes, arthropods and chordates diverged. In contrast, classic cadherins and the closely related desmosomal cadherins, as well as the more divergent protocadherins, were all amplified within the chordate lineage, resulting in a large number of subfamilies.
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in numerous genes with pronounced clustering\textsuperscript{13,14}. In humans, all six desmosomal cadherin genes are found in chromosomal region 18q12.1; three genes that encode more than 50 protocadherins are found in region 5q31–32, and many classic cadherins are organized in gene clusters\textsuperscript{13–16}. A similar gene amplification is seen in other unrelated gene clusters, such as in the *C. elegans* collagen genes. It has been proposed that the

Table 1 | Cadherin subfamilies

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<tr>
<th>Cadherin subfamily</th>
<th>Type</th>
<th>Cadherin</th>
<th>Species</th>
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*J. Petite, personal communication; † R. Cagan, personal communication (Species: Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*, Ci, *Ciona intestinalis*, Bs, *Botryllus schlosseri*, Lv, *Lytechinus variegatus*. Domains: CD, cadherin domain; CNR, cadherin-related neuronal receptor; EGF, epidermal growth factor; LG, laminin G; NCCD, non-chordate classic cadherin domain.) Uncharacterized cadherins of *Drosophila* are named according to their cytological map position (for example, DCad 102F).
I. Classic cadherins
   E-cadherin
   DE-cadherin

II. Fat-like cadherins
    DCCad76E

III. Seven-pass transmembrane cadherins
     Flamingo

IV. DCCad102F-like cadherins

V. Protein kinase cadherins
    RET

VI. Desmosomal cadherins
    Desmocollin
    Plakoglobin
    Desmoplakin

VII. Protocadherins
     Pcdh/Wd/CNR
     Plasma membrane
     Fyn

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**Figure 2 | Structural diversity of the cadherin superfamily.** Representatives of each of the seven subfamilies of cadherins are shown. Subfamilies I to VII are conserved between nematodes (Caenorhabditis elegans), arthropods (Drosophila melanogaster) and chordates (humans). Members of subfamily V are found in chordates and Drosophila, whereas cadherins of subfamilies VI and VII are at present only known in vertebrates. Binding partners for the cytoplasmic tail of cadherins have been characterized for classic cadherins (subfamily I), for desmosomal cadherins (subfamily VI), and for Pcdh/CNR/protocadherins (subfamily VII). These interacting factors are listed at the right. It was recently shown that DE-cadherin is proteolytically cleaved within the NCCD domain during maturation (arrowhead)\(^2\). (NCCD, non-chordate classic cadherin domain; EGF, epidermal growth factor; LG, Laminin G)

formation of collagen gene clusters was caused by the evolution of a complex extracellular matrix, the nematode cuticle\(^1\). The amplification of cadherins in vertebrates might be explained by the more complex tissue interactions found in humans and other vertebrates compared with invertebrates, particularly the large increase in size and complexity in the central nervous system.

**Structural basis of cell adhesion**

Although it is broadly accepted that the predominant role of cadherins is to mediate adhesive interactions between cells, the mechanism of adhesive contact formation is still a matter of intense research. Structural studies have focused on vertebrate classic cadherins. These molecules are believed to form two types of dimers. Cadherins associate laterally within the same plasma membrane to form parallel cis dimers, and cadherins protruding from adjacent plasma membranes associate in an anti-parallel fashion to form trans dimers. The structure of the first CD of E-cadherin\(^1\) and of N-cadherin\(^1\) revealed that the cadherin fold consists of a seven-strand β-sheet with its amino and carboxyl termini located at opposite ends of the molecule (FIG. 1b). The crystal structures of peptides containing the first and second CDs (CD1 and CD2) of E-cadherin\(^2\) and of N-cadherin\(^2\), indicate that calcium is central to cis-dimer formation. Each dimer associates with six calcium ions through residues that are located in the linker region between CD1 and CD2 (FIG. 3a). Single amino-acid substitutions in the calcium binding sites can disrupt cell aggregation in vivo\(^3\). Calcium binding makes CDs arrange in a rigid structure\(^4\) that is resistant to proteolysis\(^5\).

Crystallographic analysis on the first and second CDs of E-cadherin and N-cadherin have provided clues as to how cadherin molecules induce lateral clustering essential for the formation of a stable adhesive interface between adjacent cells. Although different mechanisms underlying cis dimerization have been observed in the crystal structures of different cadherin molecules\(^6\), an emerging theme is that two cadherin molecules form a cis dimer that functions as a building block for lateral clustering. These and other studies indicate that cis dimerization or more extensive lateral clustering is a prerequisite for stable cell adhesion\(^7\)-\(^9\). Although cis dimers might primarily form as homodimers, the formation of functional cis heterodimers between N- and R-cadherin has been reported\(^10\).

Adhesion between opposing cell membranes requires the formation of trans dimers (FIG. 3b). The mechanism of trans-dimer formation is, at present, controversial. Several studies indicate that trans dimers form by interactions between the amino-terminal CDs of opposing cadherin molecules\(^1\),\(^2\),\(^2\),\(^2\),\(^2\),\(^2\),\(^2\),\(^2\),\(^2\). These data are corroborated by early findings that located the homophilic binding specificity of classic cadherins within the amino-terminal CD\(^2\). On the basis of the crystal structure of the first CD from N-cadherin, a zipper model for trans dimerization was proposed that involved only the tip of the amino-terminal CD\(^2\). This model provided an early foundation for understanding the mechanics of the cell adhesion interface. However, the subsequent crystal structures of CD1 and CD2 from N-cadherin\(^1\) and E-cadherin\(^1\) did not show the adhesion interface seen in the first CD of N-cadherin. In addition, a recent biophysical study indicates a different type of trans-dimer association, in which the five CDs show variable degrees of lateral overlap, including the complete anti-parallel overlap of all five CDs (FIG. 3b)\(^1\). In the presence of calcium, the extracellular part of vertebrate classic cadherins forms a rod-like structure of about 20 nm in length with each individual CD spanning about 4.5 nm (REFS 19,20,25). Full lateral overlap of trans dimers would imply a distance between adjacent plasma membranes of 20–25 nm, a value consistent with the distance between plasma membranes at adherens junctions that is found in ultrastructural studies.

**Adhesive contacts and adherens junctions**

Two types of adhesive contacts are mediated by classic cadherins: diffuse adhesive contacts all along a cell–cell contact surface, and more discrete contacts by ultrastructurally defined adherens junctions, such as the
**ZONULA ADHERENS**
A cell–cell adherens junction that forms a circumferential belt around the apical pole of epithelial cells.

**PDZ DOMAINS**
Protein–protein interaction domain, first found in PSD-95, DLG and ZO-1.

**SH2 DOMAINS**
Src homology region 2 domains. Protein sequences of about 50 amino acids that recognize and bind sequences rich in proline.

**FILOPODIUM**
Finger-like exploratory cell extension found in crawling cells and growth cones.

**LAMELLIPODIA**
Thin sheet-like cell extension found at the leading edge of crawling cells or growth cones.

**ZONULA ADHERENS.** Diffuse adhesive contacts probably involve the oligomerization of cadherin trans dimers, as individual trans dimers provide little adhesive strength\(^2\). Adherens junctions could simply represent very large arrays of trans dimers. However, the situation seems to be more elaborate, as cadherins might not be the principal components of adherens junctions, at least in some cases. Indeed, adherens junctions can form in the absence of Hmr-1 cadherin, the only classic cadherin in *C. elegans*, or in the absence of its associated catenins\(^3\,^6\). In mouse and *Drosophila* embryos, where E-cadherin or DE-cadherin, respectively, are essential for adherens junction assembly and epithelial integrity, markedly reduced levels of these cadherins can still support the formation of normally sized adherens junctions\(^8\,^9\). These observations are inconsistent with a model in which adherens junctions simply represent a large array of cadherins and associated cytoplasmic proteins. Instead, cadherin trans dimers probably form small clusters separated by other proteins, and the density of these clusters in the adherens junctions may vary considerably without affecting the size of the adherens junction. A novel protein complex has recently been characterized that is concentrated at adherens junctions\(^40\,^42\). This complex is composed of Nectin, a transmembrane protein of the immunoglobulin superfamily that interacts with the PDZ-DOMAIN protein Afadin, which in turn can bind to Ponsin, a protein containing three SH2 DOMAINS (Fig. 4a). This complex can interact with the actin cytoskeleton. Nectin and cadherin complexes interact with each other and are recruited together to adherens junctions\(^43\). Initial functional studies indicate that Afadin is important for junctional organization and epithelial integrity\(^44\). So adherens junctions contain two complexes that interact with each other and with the actin cytoskeleton.

**Cell and tissue polarity**
Epithelial cells provide a clear example of cell polarity, with various molecules, including proteins, sorting to distinct apical and basolateral membrane domains. The crucial role of classic cadherins and their associated catenins in epithelial differentiation has been well documented, and these protein complexes seem to be broadly important for forming and maintaining epithelial tissues\(^5\,^6\). Conversely, downregulation of classic cadherins, such as E-cadherin or DE-cadherin, is often associated with a loss of epithelial morphology during normal development and in many carcinomas. The zinc-finger transcription factor Snail is important for repressing the expression of DE-cadherin and E-cadherin in non-epithelial cells\(^47\,^49\).

Epithelial cells usually form a continuous tissue structure. However, at certain times during normal development, or in experimental cell-culture models, epithelial cells have free edges that approach each other to establish new lateral contacts\(^40\,^46\). The initial contact between cells is made by FILOPODIA and LAMELLIPODIA, and such contacts are stabilized by classic cadherins. When these contacts broaden, cadherins concentrate in discrete puncta. The adhesive interactions are further stabilized through linkage of cadherins to the cytoskeleton and, eventually, by the formation of mature adherens junctions. Cadherin-mediated adhesion leads to "outside-in" signaling to initiate a number of cellular responses.

![Figure 3](image-url) **Figure 3** | **Ca\(^{2+}\)-mediated cis- and trans-dimer formation of vertebrate classic cadherins.** a | Dimer interface between two N-terminal repeats of E-cadherin domains 1 (Ecad1) and 2 (Ecad2). Each cadherin molecule binds three calcium ions that are important in the rigidification and cis-dimer association of cadherins\(^2\,^3\). b | A cis dimer consists of two cadherin molecules within the same plasma membrane that are associated laterally. The pairs of cadherin molecules from opposing cells that associate with one another are referred to as trans dimers. Different models for trans-dimer formation have been proposed that suggest different extents of lateral overlap between the extracellular regions. Red dots indicate the location of Ca\(^{2+}\) ions between adjacent CDs.
recruitment of specific cytoskeletal factors, such as the actin-associated factor Mena and other transmembrane proteins, to cell–cell contact sites.

Cadherins seem to be directly involved in maintaining cell polarity by directing the localization of the sec6/8 complex, which specifies vesicle targeting to the lateral membrane. This recruitment, and the continuous polarized delivery of specific molecular components to the lateral membrane, stabilizes and maintains the lateral membrane domain of epithelial cells and contributes to epithelial apical–basal polarity. Interestingly, in fully polarized epithelial cells, the sec6/8 complex is not found along the entire lateral membrane but is concentrated in close association with apical adherens junctions, indicating a potential direct molecular link between the cadherin–catenin complex and the vesicle-targeting machinery.

In addition to their role in apical–basal polarity, cadherin superfamily members were recently implicated in a second form of cell polarity called planar epithelial polarity (Fig. 5). This property is found in many epithelia. One example is the fly wing epithelium, where each cell polarizes its actin cytoskeleton along the proximal–distal axis, such that a bundle of actin filaments polymerizes and projects from the surface at the distal–most vertex of each cell, ultimately forming a wing hair (Fig. 5a). Genes involved in establishing the planar polarity of the wing epithelium include components of the Wnt/Frizzled signalling pathway, Frizzled and Dishevelled, and three different cadherins, Fat, Dachsous and Flamingo/Starry Night. Although the mechanism by which cadherins affect planar polarity is unknown, it was found that Flamingo/Starry Night adapts an asymmetric distribution at cell-cell contacts, becoming enriched at the proximal and distal cell surfaces (Fig. 5b). Planar polarity also influences polymerization of the microtubule cytoskeleton, manifesting itself through orientation of the mitotic spindle and thereby the axis of cell division along the body axes. Studies in both C. elegans and Drosophila implicate the Wnt/Frizzled pathway in this process. Many planar polarity genes have not been examined in this context, but it is at least clear that Flamingo/Starry Night is essential for spindle orientation.

Two other examples that highlight important roles of cadherins in generating asymmetric tissue organization are the contribution of DE-cadherin to the formation of the anterior–posterior axis in Drosophila, and the function of N-cadherin in setting up left–right asymmetry in the chick. A cell sorting process that is driven by different levels of DE-cadherin directs the oocyte to the posterior pole of the egg chamber during Drosophila oogenesis. This highly reproducible positioning event allows the oocyte to interact with a specific group of follicle cells, thereby initiating a cascade of cell interactions that are crucial for the formation of the embryonic anterior–posterior axis. Disruption of the function of N-cadherin during chick gastrulation leads to a random orientation of the heart along the left–right axis. Asymmetric N-cadherin expression and cell movements that prefigure the position of the heart and other organs along the left–right axis are seen during gastrulation. How N-cadherin contributes to these asymmetric cell movements remains a mystery.

Cell movement

Many of the changes in cell shape or movement observed during development occur while cells are in direct contact and require, therefore, dynamic changes in adhesive interactions. These changes may play a permissive role, as the release of adhesion is important for the relative movement of cells that are in contact. However, adhesive interactions also directly promote movement, as traction must be generated between cells for cell rearrangements to occur in solid tissues. To determine whether changes in cadherin activity play a permissive or a more active role can be difficult, as illustrated by the analysis of C-cadherin function during...
convergent extension movements in *Xenopus* gastrulation. In this process, cells move towards the dorsal midline of the embryo, thereby rearranging by cell intercalation, leading to an extension of the embryo along the anterior–posterior axis (FIG. 6a). Adhesion mediated by the classic cadherin C-cadherin must be reduced to permit these movements to occur. However, the disruption of C-cadherin activity causes defects not only during gastrulation movements, but also in tissue structure, raising the possibility that the disruptions in cell movement might be a secondary consequence of a compromised cell architecture. Similar difficulties have emerged from the analysis of DE-cadherin in embryonic morphogenesis where its role in epithelial maintenance might mask a function in promoting cell rearrangements.

Paraxial protocadherin (PAPC) seems to be directly involved in convergent extension in *Xenopus* and zebrafish embryos, where it is expressed in the mesoderm during gastrulation. PAPC, which can promote homotypic cell adhesion, is required for convergent extension of the mesoderm. Notably, overexpression of PAPC can promote convergent extension under certain experimental conditions. These findings argue that PAPC acts as an adhesion receptor that directly promotes cell movement, perhaps providing traction needed for cell motility. Alternative and non-exclusive possibilities are that PAPC is primarily a signaling receptor, as was suggested for other protocadherins. PAPC activity might also generate the tissue polarization that is observed during convergent extension (FIG. 6a), functioning similarly to the activity of other cadherins in planar epithelial polarization, outlined above. Intriguingly, cell polarization during convergent extension resembles planar polarity in that it also requires Wnt/Planar polarity.

The requirement for DE-cadherin in cell migration during Drosophila oogenesis is a convincing example for a direct role of classic cadherins in cell migration on a cellular substrate. DE-cadherin is involved in the migration of a small group of somatic cells, the 'border' cells, on the surface of the much larger germline cells (FIG. 6b). It is required in both the somatic and germline cells for this movement to occur. DE-cadherin is not required for the formation of the border cell cluster and, more importantly, is not required for maintaining integrity of the border cell cluster during migration. In the case of integrin-based cell migration, it was shown that intermediate levels of adhesion to the substrate promote maximal migration speed, with both positive and negative deviations slowing or halting motility. Similarly, reduction in the level of DE-cadherin reduces the speed of border cell migration, indicating that DE-cadherin might not have just a permissive role, but might be the key adhesion molecule that provides traction for border cells to travel over germline cells.

**Organization of the nervous system**

Various cadherins are expressed in the nervous system in complex patterns. The first example was N-cadherin, which is broadly expressed in the neuroepithelium, beginning at neurulation. This expression pattern led to the speculation that N-cadherin might be critical for the segregation of neural and epidermal tissues during neural tube formation. However, an essential role in neurulation was disproved by the knockout of mouse *N-cadherin*. After neurulation, but before neuronal differentiation, many classic cadherins, including N-cadherin, are expressed in the developing central nervous system (CNS) in a region-specific manner that often coincides with morphological boundaries. The most direct evidence that these cadherins contribute to the subdivision of the neuroepithelium has come from analysis of the *Xenopus* type II classic cadherin F-cadherin. The expression of F-cadherin confines neuroepithelial cells to the sulcus limitans, a region separating the dorsal and ventral halves of the caudal neural tube. One apparent consequence of F-cadherin expression in the dorsal neural tube is the expression of a diffuse adhesive contact, or an adherens junction, such as the zonula adherens, that can be seen in electron micrographs as an electron-dense specialization of the plasma membrane. Epithelial sheets are obviously different from a cellular substrate. DE-cadherin is involved in the migration of a small group of somatic cells, the 'border' cells, on the surface of the much larger germline cells (FIG. 6b). It is required in both the somatic and germline cells for this movement to occur. DE-cadherin is not required for the formation of the border cell cluster and, more importantly, is not required for maintaining integrity of the border cell cluster during migration. In the case of integrin-based cell migration, it was shown that intermediate levels of adhesion to the substrate promote maximal migration speed, with both positive and negative deviations slowing or halting motility. Similarly, reduction in the level of DE-cadherin reduces the speed of border cell migration, indicating that DE-cadherin might not have just a permissive role, but might be the key adhesion molecule that provides traction for border cells to travel over germline cells.
the sulcus limitans is that these cells remain a coherent group and do not participate in the extensive cell rearrangements that take place during neurulation.

Protocadherins also contribute to CNS regionalization by controlling the migration of neurons that will organize into different cortical layers during brain morphogenesis. Although cadherins, including protocadherins, are generally viewed as homophilic adhesion molecules, recent work indicates that the Pcdh/CNR protocadherins might also function as receptors for co-receptors for extracellular ligands in the brain. This work began with studies of the secreted molecule Reelin, identified because mutant mice have a marked behavioural disorder. Two protein families have been shown to function as reelin receptors, perhaps as a heteromeric complex: members of the LDL-receptor-related protein family, which couple to the cytoplasmic adaptor protein mDab1; and members of the Pcdh/CNR family, which bind the non-receptor tyrosine kinase Fyn. As Pcdh/CNR protocadherins show considerable molecular diversity and differential expression patterns within local brain areas, it is possible that Reelin receptor complexes that contain different Pcdh/CNR protocadherins are instructive in positioning and differentiating neuronal sub-populations within the cortex.

Classic cadherins are also important during the outgrowth of neurites and during axonal patterning and fasciculation. Early studies that indicated that N-cadherin can function as a substrate for neurite extension in cultured cells were reinforced by the finding that N-cadherin is required for the normal outgrowth and guidance of retinal axons. In Drosophila, DN-cadherin is the only classic cadherin expressed in the developing embryonic CNS. Null mutations in DN-cadherin and the Drosophila catenin gene armadillo affect axon outgrowth, although in a mild fashion, with many axons finding their targets appropriately. In this respect, cadherins resemble various other axon guidance cues that direct axon outgrowth in a combinatorial fashion, with individual cues having subtle functions. In addition, it was recently found that the patterning of dendrites that extend from Drosophila sensory neurons requires Flamingo/Starry Night.

Both classic and protocadherins localize to synapses, indicating that they may contribute to the generation of adhesive specificity needed to build complex neural networks. The synapse is an adhesive contact between two neurons, with the transmitter release zone framed by adhesion junctions that are ultrastructurally similar to epithelial adhesion junctions, and that share with them the cadherin–catenin complex as a principal molecular component. In synapses, as in epithelial cells, adhesion junctions are closely associated with vesicle-release zones. It is believed that classic cadherins are important during synaptic adhesion, whereas the adhesive role of protocadherins at synapses remains to be clarified. Recent intriguing evidence indicates that synaptic activity can change the distribution and adhesive state of N-cadherin. Cadherins, in turn, can influence synaptic activity. These findings indicate an intimate relationship between synaptic adhesion and activity, raising the possibility that cadherins are important regulators of synaptic plasticity and activity modulation.

Some classic cadherins, such as cadherin-6, are expressed in groups of neurons that form neural circuits, indicating that cadherins might functionally integrate such circuits. Protocadherins are also expressed in divergent and restricted patterns in the CNS, indicating that they might have a function in integrating neural circuits. Moreover, the protocadherin genes Pcdha, Pdhd3 and Pdhd4 can give rise to over 50 protocadherins and the Pcdha/CNR proteins have a differential expression pattern within individual brain areas. These findings raise the possibility that a ‘cadherin code’ exists, which could identify individual neurons and their synaptic contacts, although many adhesion molecules show provocative similarities to the gene organization of immunoglobulins or T-cell receptors, which has led to the proposal that green rearrangement might function in determining protocadherin expression patterns, a speculation that remains to be proved.

The future

The analysis of cadherins emphasizes the similarities between embryonic and neural morphogenesis. Cadherins have emerged as the predominant group of cell–cell adhesion molecules involved in embryonic morphogenesis, determining cell and tissue architecture, and controlling dynamic changes in cell shape and position. The role of individual cadherins in several specific morphogenetic processes has been determined, which...
will now allow the study of how the adhesive activity of cadherins is modulated by cell signaling to facilitate coordinated cell behaviour. Cadherins are also important
tant during neural morphogenesis, although the func
tional significance of cadherins in neural development remains less well understood. One important challenge will be to determine the exact mechanism underlying
the specificity and stability of cadherin-mediated cell-cell adhesion, and to explore the variation in adhe
sive mechanisms and cellular responses of different

types of cadherins. A second important challenge will be to substantiate the conjecture that cadherins provide
an adhesive code that controls synaptic specificity.
Elucidating whether and how different families of adhe
sion receptors cooperate in this process will represent an
enormous advance in our understanding of complex
neural network formation.
polycystic kidney phenotype by altering Frizzled signaling. 
Development 125, 655-666 (1999).
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64. Experiments in this paper indicate an important role for a protocadherin in the convergent extension movements during frog gastrulation.
70. This paper documents a cadherin-dependent cell migration process.
76. Protocadherins of the CNF family are identified as receptors of the extracellular matrix protein Reelin, an interaction that might contribute to the migration and differentiation of neurons within the brain cortex.
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The canonical Wg and JNK signaling cascades collaborate to promote both dorsal closure and ventral patterning

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SUMMARY

Elaboration of the *Drosophila* body plan depends on a series of cell-identity decisions and morphogenetic movements regulated by intercellular signals. For example, Jun N-terminal kinase signaling regulates cell fate decisions and morphogenesis during dorsal closure, while Wingless signaling regulates segmental patterning of the larval cuticle via Armadillo. wingless or armadillo mutant embryos secrete a lawn of ventral denticles; armadillo mutants also exhibit dorsal closure defects. We found that mutations in puckered, a phosphatase that antagonizes Jun N-terminal kinase, suppress in a dose-sensitive manner both the dorsal and ventral armadillo cuticle defects. Furthermore, we found that activation of the Jun N-terminal kinase signaling pathway suppresses armadillo-associated defects. Jun N-terminal kinase signaling promotes dorsal closure, in part, by regulating *decapentaplegic* expression in the dorsal epidermis. We demonstrate that Wingless signaling is also required to activate *decapentaplegic* expression and to coordinate cell shape changes during dorsal closure. Together, these results demonstrate that MAP-Kinase and Wingless signaling cooperate in both the dorsal and ventral epidermis, and suggest that Wingless may activate both the Wingless and the Jun N-terminal kinase signaling cascades.

Key words: Armadillo, Dorsal closure, Segment polarity, Puckered, Wingless, JNK, Wnt, β-catenin, *Drosophila*

INTRODUCTION

Proper patterning of multicellular organisms depends on stringent regulation of cell-cell signaling. Members of the Wnt/Wingless (Wg) family of secreted glycoproteins direct cell fates in both insects and vertebrates (reviewed in Wodarz and Nusse, 1998). Genetic studies revealed many of the genes required for Wg signaling in *Drosophila*. Mutations in several of these, including *armadillo* (*arm*), were first identified in a screen for genes whose zygotic expression is required for embryonic viability and proper patterning of the larval cuticle (Nusslein-Volhard and Wieschaus, 1980). *arm* mutant embryos exhibit segment polarity defects characterized by secretion of a ventral lawn of denticles and a concomitant loss of naked cuticle. This phenotype resembles that of wg mutants, because Arm functions in the transduction of Wg signal.

Arm is the *Drosophila* ortholog of human β-catenin (β-cat). Both are found associated with cell-cell adherens junctions, in the cytoplasm and in nuclei. Wg/Wnt signaling elicits a cellular response by regulating the free pool of Arm/β-cat. In the absence of Wg/Wnt signal, cytoplasmic Arm/β-cat is rapidly degraded via a proteasome-mediated pathway (reviewed in Polakis, 1999). Cells of the *Drosophila* epidermis that receive Wg signal accumulate Arm in the cytoplasm and nucleus (reviewed in Wodarz and Nusse, 1998). This depends upon Wg binding cell surface receptors of the Frizzled (Fz) family, the activation of Dishevelled (Dsh) and subsequent inactivation of Zeste white-3 (Zw3, the *Drosophila* ortholog of glycogen synthase kinase-3β). From studies of mammals, two additional proteins, APC and Axin, were implicated in regulating β-cat stability (reviewed in Polakis, 1999). Members of the TCF/LEF family of transcription factors are also required for Wnt/Wg signal transduction (reviewed in Wodarz and Nusse, 1998). TCF/LEF transcription factors bind DNA, and recruit Arm/β-cat as a co-activator, thus activating Wg/Wnt-responsive genes. Therefore, Wg/Wnt signaling regulates cell fate choices directly by altering the patterns of gene expression.

In *Drosophila*, upstream components of the Wg pathway are also required during the establishment of planar polarity, the process whereby epithelial cells acquire positional information relative to the body axes of the animal. For example, both Fz and Dsh are required to coordinate the proximal-to-distal orientation of actin-based wing hairs (reviewed in Shulman et al., 1998). Most tests have suggested that planar polarity is independent of Arm function, while genetic and biochemical studies suggest that the Jun N-terminal kinase (JNK) signaling pathway functions downstream of Fz and Dsh to establish planar polarity (reviewed in Boutros and Modzik, 1999). It remains unclear whether Fz is activated by Wg, or any Wnt, during the establishment of planar polarity. It has been
suggested that JNK signaling and the canonical Wg pathway function as alternate signal transduction pathways downstream of Fz, with Dsh functioning as a branch point from which different cellular processes are elicited in a tissue-specific manner.

In *Drosophila*, the JNK pathway is best known for its role in dorsal closure (reviewed in Noselli and Agnes, 1999). During stage 13 of embryogenesis, two lateral epidermal sheets migrate toward the dorsal midline where they fuse. Mutations in the *Drosophila* orthologs of JNK kinase (JNKK; Hemipterous; Hep; Glise et al., 1995), JNK (Basket; Bsk; Riesgo-Escovar et al., 1996; Sluss et al., 1996), as well as the transcription factors Fos (Kayak; Kay; Riesgo-Escovar and Hafen, 1997; Zeitlinger et al., 1997) and Djin (Hou et al., 1997; Kockel et al., 1997; Riesgo-Escovar and Hafen, 1997), block dorsal closure. Other regulators of dorsal closure include Misshapen, a Ste20 relative (Paricio et al., 1999; Su et al., 1998), Puckered (Puc), a VHI-like phosphatase (Martin-Blanco et al., 1998), and Decapentaplegic (Dpp), a TGFB relative (reviewed in Noselli and Agnes, 1999). Both Puc and Dpp are activated in the dorsalmost epidermal cells by JNK signaling (reviewed in Noselli and Agnes, 1999). Dpp then acts over several cell diameters to coordinate cell shape changes throughout the epidermis. Puc, in contrast, antagonizes JNK signaling in cells adjacent to the leading edge. The ligand that initiates JNK signaling in this context remains to be identified.

Mutations in APC and β-catenin play roles in both colorectal cancer and melanoma (reviewed in Polakis, 1999), but these mutations fail to account for all cases, suggesting that other Wnt signaling antagonists might be involved in oncogenesis. To identify antagonists that function in embryogenesis and/or oncogenesis, a modifier screen for suppressors of the arm zygotic phenotype was performed (Cox et al., 2000). In the course of this screen, an unexpected connection between JNK and Wg signaling was discovered. Previous models suggested that distinct signaling pathways operate in the ventral and dorsal epidermis, with JNK signaling coordinating dorsal closure and Wg signaling acting through Arm to establish segment polarity in the ventral epidermis. Here, we demonstrate that mutations in *puc*, a known negative regulator of JNK signaling, suppress both the dorsal closure and ventral segment polarity defects associated with a decrease in Wg signaling. Furthermore, we present evidence that downstream components of the Wg signaling pathway, like Arm and DTCF, act together with JNK signaling pathways in both ventral patterning and dorsal closure. Our data are consistent with a model whereby Wg activates separate, yet parallel, signaling cascades that are required to promote dorsal closure and establish ventral pattern.

**MATERIALS AND METHODS**

**Fly stocks and phenotypic analysis**

The wild-type stock was Canton S. Mutants used are described at http://flybase.bio.indiana.edu. Unless otherwise noted, *arm*(* arm*^{P37Y}), *puc*(* puc*^{251.1}), *wg^{wre}=wg^{P4D(2)DE} and *wg^{em}=wg^{G22}, 2w3^{M1-1} and *dsh*^{79} germline clones were generated as in Pefer et al. (1994). Stocks were obtained as follows: *puc*^{251.1}, *Djin*^{2} and *kay*^{1}, the Bloomington *Drosophila* stock center; *puc*^{91} and *puc*^{380}, A. Martinez Arias; UAS-*dpp*, UAS-*tkv*{A}, UAS-*kv*^{253D}, UAS-*DTak*^{WT3}, UAS-*DTak*{1} and UAS-*DTak*^{D(1-1)}, M. B. O'Connor; UAS-*Bsk* and UAS-*Hep*, M. Mlodzik; LE-*Gal4*, S. Noselli; UAS-*Wg*, A. Bejsovec; *arm*-*Gal4*>>VP16 and β-tubulin-flp, J. P. Vincent and D. St. Johnston. To express *Gal4*>>VP16, *arm*-*Gal4*>>VP16/TM3 females were mated to β-tubulin-flp males. Non-TM3 F1 males were collected and mated en masse to UAS females.

**In situ hybridization and immunofluorescence**

Decorbyorated embryos were fixed for 5 minutes with 1:1 37% formaldehyde/heptane, devitellinized with 1:1 heptane/methanol, postfixed for 15 minutes in 1.85% formaldehyde/PBT (1× PBS + 0.1% Triton X-100), washed extensively with PBT, and transferred to 1× HYB buffer (50% formamide, 5× SSC, 100 μg/ml salmon sperm DNA, 100 μg/ml E. coli tRNA, 50 μg/ml heparin, 0.1% Tween-20, pH 4.5) for 1 hour at 70°C. Embryos were incubated overnight at 70°C with heat-denatured digoxigenin-labeled antisense RNA, washed at 70°C once each with 1× HYB, 2:1 HYB/PBT and 1:2 HYB/PBT, four times with PBT, incubated for 1 hour at 25°C with alkaline phosphatase-conjugated anti-digoxigenin (1:2,000; Boehringer Mannheim), washed with PBT, and equilibrated with 1× AP buffer (100 mM Tris (pH 9.5), 100 mM NaCl, 50 mM MgCl₂, 0.1% Tween-20). Transcripts were visualized with NBT and BCIP.

**RESULTS**

**puc mutations suppress loss of Arm function**

Wg signaling is required to establish posterior cell fates in each larval epidermal segment. In wild-type embryos, anterior cells of each segment secrete cuticle covered with denticles, while posterior cells secrete naked cuticle (Fig. 1A). *arm* mutants, like other mutations disturbing Wg signaling (Nusslein-Volhard and Wieschaus, 1980), exhibit a lawn of ventral denticles and thus loss of naked cuticle (Fig. 1B). In addition, strong hypomorphic or null *arm* alleles, like *arm*^{P37} and *arm*^{91}, have a dorsal hole, suggesting problems during dorsal closure. Thus, Arm is required during establishment of segment polarity and during dorsal closure.

During a screen for suppressors of *arm*′s embryonic phenotype (Cox et al., 2000), we found that one suppressor was a P-element-induced allele of *puc* (*puc*). *puc* encodes a VH1-like phosphatase that acts as a negative-feedback regulator of the JNK signaling pathway during dorsal closure (Martin-Blanco et al., 1998). *puc*^{251.1} is a strong *puc* allele, exhibiting the characteristic “puckering” of the dorsal epidermis (Fig. 1E; unless noted, *puc* henceforth refers to *puc*^{251.1}), and results from the insertion of a P-element enhancer trap in intron 2 (Martin-Blanco et al., 1998).

Heterozygosity for *puc*′s (*puc*^{251.1}/+), strongly suppresses *arm*′s dorsal closure defects, while ventral patterning defects are moderately suppressed (Fig. 1C). *arm*′s *puc*^{251.1} double mutants have a more pronounced suppression of the segment polarity phenotype, with naked cuticle reappearing (arrowheads in Fig. 1D). In addition, *arm*′s *puc*^{251.1} double mutants have a novel dorsal phenotype, characterized by loss of dorsal cuticle (+ in Fig. 1D). Another strong *puc* allele, *puc*^{91} (Martin-Blanco et al., 1998), suppresses *arm* to a similar extent, while weak alleles like *puc*^{90} fail to suppress either the dorsal or ventral defects (data not shown). Thus, a reduction in Puc activity suppresses, in a dose-sensitive manner, both the dorsal and ventral cuticle phenotypes of *arm*.
suggest that Puc normally antagonizes Wg signaling and that of the denticle belts in the ventral epidermis, the cuticle pattern of puc mutants was examined. In puc mutants, the denticle belts are narrowed in the anteroposterior (AP) axis with the strongest narrowing at the ventral midline (compare Fig. 2A to B). This phenotype is similar to that caused by weak activation of the Wg signaling pathway (Pai et al., 1997), further suggesting that Puc normally antagonizes Wg signaling in the ventral epidermis.

The only known biochemical role for Puc is as an antagonist of JNK activity (Martin-Blanco et al., 1998). While the JNK pathway has only been shown to act during dorsal closure, hemipterous mRNA is expressed uniformly throughout the epidermis (Glise et al., 1995) and basket mRNA is expressed throughout the epidermis but enriched at the leading edge (Riego Escovar et al., 1996; Sluss et al., 1996). To test whether Puc may affect ventral patterning by regulating JNK signaling ventrally, we first examined the expression of the pucA251.1 enhancer trap, a JNK target gene, in the ventral epidermis of puc mutants. In phenotypically wild-type embryos, expression of β-gal, driven by puc enhancer traps, is completely restricted to the dorsalmost epidermal cells (Fig. 2C), where puc mRNA is also normally enriched—no expression is seen in the ventral epidermis (Fig. 2C; Glise and Noselli, 1997; Martin-Blanco et al., 1998; Ring and Martinez Arias, 1993). In contrast in puc homozygotes, we found significant activation of this JNK target gene in the ventral epidermis, in addition to its previously reported activation in cells adjacent to the leading edge (Fig. 2D; Glise and Noselli, 1997; Martin-Blanco et al., 1998; Ring and Martinez Arias, 1993). The pucA251.1 enhancer trap was activated in intermittent stripes of cells extending from the dorsal epidermis to the ventral midline (Fig. 2E). These stripes are at the anterior margin of the presumptive denticle belts (brackets in Fig. 2F) and co-localize, in part, with Engrailed in the posterior compartment of each segment (data not shown). In the ventral epidermis, substantial activation of the enhancer trap was also observed near the ventral midline with the strongest expression just posterior to and overlapping the prospective denticle belts (Fig. 2F). Ectopic expression of the enhancer trap correlates well with the narrowing of denticle belts in puc mutants; the

**Puc antagonizes Wg signaling in ventral patterning, perhaps via the JNK pathway**

Puc was previously reported to specifically affect dorsal closure (Martin-Blanco et al., 1998; Ring and Martinez Arias, 1993). To assess whether loss of Puc function also affects cell fate choices in the ventral epidermis, the cuticle pattern of puc mutants was examined. In puc mutants, the denticle belts are narrowed in the anteroposterior (AP) axis with the strongest narrowing at the ventral midline (compare Fig. 2A to B). This phenotype is similar to that caused by weak activation of the Wg signaling pathway (Pai et al., 1997), further suggesting that Puc normally antagonizes Wg signaling in the ventral epidermis.

**puc mutations suppress mutations in Wg signaling**

To assess whether this suppression was specific to arm, the cuticle phenotypes of puc:dTCF2, wgweak:puc and wgnull:puc double mutants were examined. Loss of Puc function suppressed the ventral segment polarity defects of both dTCF2 (Fig. 1F versus G) and a weak wg mutant (wgweak; Fig. 1H versus I). In contrast, loss of Puc function did not suppress the segment polarity defects of wgnull alleles (Fig. 1J versus K). puc:dTCF2, wgweak:puc and wgnull:puc double mutants all also have a reduction in dorsal epidermis (data not shown) similar to that of arm:puc double mutants (Fig. 1D). These data suggest that Puc normally antagonizes Wg signaling and

**Fig. 1. Inactivation of Puc suppresses defects in Wg signaling. Ventral views (except E) of cuticles, with anterior up. (A) Wild type; (B) armXP35/Y. Note the hole in the dorsal cuticle (arrow). (C) armXP35/Y;pucA251.1/y. Note increase in cuticle length and lack of a dorsal hole—an anterior hole remains (arrow). (D) armXP35/Y.pucA251.1, * denotes loss of dorsal cuticle. Arrowheads (D,G,J) denote patches of naked cuticle. (E) pucA251.1. No suppression is seen.**

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**Fig. 2.** Puc antagonizes Wg and negatively regulates a JNK reporter during ventral patterning. (A) Ventral view of wild-type cuticle; (B) ventral view of puc\(^{A251/1}\). Note narrowing of denticle belts and occasional gaps in denticles at the midline. (C-H) Embryos double-labeled with anti-β-gal (green: indicates expression of the puc enhancer trap), and anti-PTyr (red; labels adherens junctions). (C) Lateral view of stage 12 wild-type (puc\(^{A251/1}\))/+ embryo. The enhancer trap is expressed exclusively in cells of the leading edge of the dorsal epidermis (arrowhead) and not expressed at all in the ventral epidermis. (D) Lateral view of stage 12 puc mutant (puc\(^{A251/1}\)/puc\(^{A251/1}\)) embryo. Note expansion of enhancer trap expression into several rows of cells adjacent to the leading edge (arrowhead). (E,F) (close-up) Lateral views of stage 14 puc\(^{A251/1}\) embryo. β-gal expression is found in the ventral epidermis where it is never observed in a wild-type embryo. Brackets (F) denote prospective denticles belts. Note β-gal expression both near the ventral midline and in a row of cells at the anterior edge of the developing denticles belts (arrowhead); these cells will be converted from denticles to naked cuticle fates. (G) Ventral view of stage 14 arm\(^{Xp33}Y\)/puc\(^{A251/1}\) embryo. Expanded stripes of enhancer trap expression (arrowheads) correlate with reappearance of naked cuticle (Fig. 1D). Brackets denote prospective denticles belts. (H) Ventral view of stage 14 wg\(^{R222}\)/puc\(^{A251/1}\) embryo. Segmental expression of the enhancer trap is diminished (arrowhead). Anterior is to the left in all panels.

A correlation between expression of the puc enhancer trap and conversion to naked cuticle fates was also observed in arm/puc double mutants. Domains of β-gal expression up to three cells wide appear in the ventral epidermis (arrowheads in Fig. 2G). Double labeling for anti-phosphotyrosine (PTyr), to visualize developing denticles, and β-gal demonstrated that cells expressing puc\(^{A251/1}\) enhancer trap differentiate into naked cuticle (Fig. 2G). In wg\(^{null}\)/puc embryos, in contrast, while cells along the midline continue to express β-gal, the segmental stripes of β-gal are diminished (Fig. 2H). This reduction in enhancer trap expression correlates with the failure of wg\(^{null}\)/puc double mutants to secrete naked cuticle (Fig. 1K). One possible explanation of this observation is that Wg signaling promotes puc expression; however, the loss of puc-expressing cells might also result from the ectopic apoptosis that occurs in wg mutants.

To further test whether puc mutations affect ventral patterning via activation of JNK signaling, we directly tested whether activation of the pathway by elevated expression of the kinases mimics the effects of puc mutations. We mis-expressed Hap or Bsk, the Drosophila JNKK and JNK orthologs (reviewed in Noselli and Agnes, 1999), in an arm mutant background using the GAL4-UAS system (Brand and Perrimon, 1993). Ubiquitous expression of either Bsk and Hap (Fig. 3C,D) results in a partial suppression of the arm phenotype, to a degree similar to the suppression by puc heterozygosity (Fig. 1C). The size of the embryo is expanded and dorsal closure defects are often alleviated, but naked cuticle is not restored. Expression of the Drosophila TAK1 ortholog DTak, a putative JNK kinase (Takatsu et al., 2000), had a more dramatic effect. Mis-expression of DTak in even numbered segments of the developing arm epidermis using the paired-GAL4 driver resulted in the reappearance of naked cuticle in those segments (Fig. 3F). Furthermore, misexpression of DTak in a wild-type background using the same paired-GAL4 driver resulted in narrowing of the denticles belts (Fig. 3G), similar to that caused by weak activation of the Wg pathway (Pai et al., 1997) or by loss of Puc function (Fig. 2B). These data are consistent with the idea that Puc's effects on the ventral pattern occur via regulation of the JNK signaling pathway, though other mechanisms, such as a direct role for Puc in the canonical Wg pathway, remain possible.

In both puc and arm/puc mutants, cells that activate the JNK pathway acquire naked cuticle cell fates. This is consistent with the hypothesis that Puc normally antagonizes production of naked cuticle in the ventral epidermis by suppressing JNK signaling. It should be noted, however, that loss-of-function mutations in JNK pathway components, including bsk, Djun and kayak (Dfos), do not substantially alter the ventral denticles pattern (data not shown). This suggests that, if the JNK pathway plays a role in ventral patterning, it may function semiredundantly with other MAPK signaling pathways, as it does in planar polarity in the eye (Boutros et al., 1998). This could also explain why DTak has a stronger effect, as it may be upstream of more than one MAPK module.

We also tested a second means by which Puc could influence ventral patterning. The EGF-receptor (EGFR), acting via the ras-ERK pathway, promotes development of denticles cell fates and thus antagonizes Wg signaling, which promotes naked cuticle (O'Keefe et al., 1997; Szuts et al., 1997). If Puc upregulated this pathway, perhaps via effects on ERK, this
might explain Puc’s effects on ventral patterning. Therefore, we
tested whether heterozygosity or homozygosity for loss-of-
function mutations in the EGFR pathway suppressed arm.
Mutations in the ligand vein\(^{1,2}\), the receptor EGFR\(^{1,8}\)
(heterozygotes only), rolled (MAPK/ERK) and ras85B\(^{1,8}\)
did not significantly suppress either the dorsal or ventral arm
phenotypes (Cox et al., 2000; data not shown). As
heterozygosity for these mutations suppresses other
phenotypes resulting from activation of MAPK signaling (e.g.,
Simon et al., 1991), these results suggest that Puc is unlikely
to influence ventral pattern via the EGFR pathway, a result
consistent with Puc’s inability to regulate ERK activity
(Martin-Blanco et al., 1998).

**Wg signaling regulates dpp expression at the leading edge**

Previous studies identified a role for JNK signaling during
dorsal closure. The unexpected interaction between Puc and
Wg signaling in ventral patterning, as well as puc’s suppression
of arm’s dorsal closure defect (Fig. 1C), led us to examine the
potential role of Wg signaling during dorsal closure. Cells of
the leading edge, which initiate dorsal closure, activate a
specific transcriptional program. Thus, we assessed whether
Wg signaling plays a role in dorsal closure by regulating expression of dpp, a TGF-\(\beta\) family member. Dpp is expressed
in cells of the presumptive leading edge before germband
retraction and by leading edge cells after germband retraction
(Fig. 4A). It is thought to promote dorsal closure by initiating
cell elongation in the dorsoventral (DV) axis (reviewed in
Noselli and Agnes, 1999). JNK signaling is essential for
continued dpp expression at the leading edge (reviewed in
Noselli and Agnes, 1999); in embryos mutant for the JNK
pathway, dpp expression in these cells is lost (Fig. 4B,C),
resulting in a failure to complete dorsal closure (Fig. 4).

We thus examined dpp expression in embryos where Wg
signaling was compromised. In embryos mutant for strong
alleles of wg (wg\(^{G22}\) or wg\(^{CM4}\), dpp expression was initiated at
the leading edge but then decayed; by the onset of dorsal closure,
dpp expression was strongly reduced or absent (Fig. 4D). Similar
results were seen in embryos zygotically mutant for strong
(arm\(^{XP33}\); Fig. 4F) or null arm alleles (arm\(^{T035}\); data not shown).

To further reduce Arm function, we made embryos maternally
and zygotically mutant for arm\(^{Mm19}\), an allele that preferentially
eliminates Arm function in Wg signaling. In these embryos,
leading edge expression of dpp was reduced substantially, with
no detectable expression observed during dorsal closure (Fig.
4G). dpp expression was also lost from lateral epidermal cells
as well as from the foregut and hindgut, both ectodermal
tissues. In contrast, dpp expression remained in several other
tissues, including the midgut (white arrows) and clypealabrum.

Similar loss of dpp expression both at the leading edge
and in the lateral epidermis was seen in embryos
maternally and zygotically mutant for a strong dsh
allele, dsh\(^{75}\) (Fig. 4E). Together, these results suggest
that Wg signaling promotes dpp expression in leading edge
cells after germband retraction. Furthermore, Dsh and
Arm are required for dpp expression in other
ectodermal tissues.

dTCF encodes a transcription factor that co-
operates with Arm to activate Wg target genes. dTCF
is maternally contributed to the embryo; to overcome
this maternal pool, a N-terminally truncated form of
dTCF, dTCF\(\Delta\), which functions as a constitutive
repressor of Wg target genes (Cavallo et al., 1998),
was overexpressed in the developing epidermis. In
such embryos, dpp expression in the dorsoalmost
epidermal cells was attenuated but not eliminated
(Fig. 4H); expression of dpp in the lateral epidermis
was also strongly reduced. This suggests that dTCF
may also be required for Wg’s activation of dpp.

The puc enhancer trap is a second JNK target gene
activated in leading edge cells (Fig. 5A; reviewed in
Noselli and Agnes, 1999). Therefore, we examined
its expression in several wg-class mutants — zygotic
arm (Fig. 5B,C), wg\(^{null}\) (Fig. 5D) and embryos
maternally and zygotically mutant for arm\(^{Mm19}\)
(Fig. 5E) or dsh\(^{75}\) (Fig. 5F). In all, enhancer trap
expression was reduced or lost in a subset of leading
dge cells, although detectable expression of the
enhancer trap always remained. To further examine
the role Wg signaling plays in regulating the puc
enhancer trap, we examined arm\(\Delta\);puc and wg\(^{null}\);puc
double mutant embryos. In puc single mutant
embryos, puc enhancer trap expression expands
during dorsal closure into additional lateral cells
(Glise and Noselli, 1997; King and Martinez Arias,
Fig. 4. Wg signaling is required for dpp expression and dorsal closure. (A-H) Lateral views of stage 13 embryos labeled for dpp mRNA, with anterior at left. Note dpp at the leading edge (black arrows), in the midgut (white arrows) and in the lateral epidermis (black arrowheads). (A) Wild type; (B) Djwi'; (C) UAS-puclarm-GAL4::VP 16; (D) wgCX4; (E) maternal and zygotic dsh75; (F) armXP3; (G) maternal and zygotic armxM19; (H) UAS-dTCFAN;arm-GAL4::VP16. Note loss of dpp mRNA at the leading edge (black arrows) while midgut expression (white arrows) remains. (I-P) Cuticles. (I) Dorsal view of wild type. (J) UAS-puc/arm-GAL4::VP16. The dorsal surface is completely open, with edges of the large dorsal hole indicated by arrows. (K,L) wgG22. Many wg embryos (K) are dorsally closed but exhibit severe defects in dorsal patterning; note abnormal cuticular structures (arrowhead). (L) 18% are mildly dorsally open. In K-P, the arrow indicates the posterior extent of the dorsal hole. (M) Maternal and zygotic dshi75 mutant; (N) armXP3 mutant; (O) maternal and zygotic armxM19 mutant; (P) UAS-dTCFAN;arm-GAL4::VP16 mutant. Note dorsal closure defects.

1993; Fig. 5G), due to hyperactivation of the JNK pathway. In arm:puc and wgpumu:puc double mutants, this expansion was attenuated (Fig. 5H,l), consistent with a role for Wg in regulating puc expression in dorsal epidermal cells.

Constitutive activation of Wg signaling drives ectopic dpp expression

These data suggest that both the JNK and canonical Wg pathways regulate dpp in leading edge cells. When the JNK pathway is ectopically activated, as occurs in puc mutants, dpp expression expands into a subset of the more lateral epidermal cells (Fig. 6A versus B; reviewed in Noselli and Agnes, 1999). We thus examined whether ectopic activation of the Wg pathway also expands the domain of dpp expression. We activated the Wg pathway by removing maternal and zygotic Zw3 (Fig. 6C,D) or by mis-expressing Wg (data not shown) or a constitutively active form of Arm (data not shown) throughout the dorsal epidermis. In all cases, dpp expression expanded from the leading edge into more lateral cells.

Ectopic activation of Wg signaling also mimics the effects of puc on the dorsal cuticle. puc mutants exhibit a characteristic 'puckering' of the anterior and posterior epidermis towards the dorsal surface, as well as defects in dorsal hair diversity and patterning (Fig. 6E versus F; Martin-Blanco et al., 1998; Ring and Martinez Arias, 1993). Activation of Wg signaling throughout the dorsal epidermis, by ubiquitously expressing Wg (Fig. 6G) or ArmS10 (Fig. 6I), or in zw3 mutants (Fig. 6H), results in a very similar disruption of the dorsal cuticle, including 'puckering', dorsal hair patterning and identity defects (4"only", and 'bald' scars along the dorsal midline. Thus, overactivation of either JNK or Wg signaling pathways has a similar effect upon patterning of the dorsal epidermis.

Activation of JNK or Dpp signaling in the leading edge rescues dorsal closure of arm mutants

Misexpression of Hep throughout the epidermis suppresses the phenotype of arm (Fig. 3D). To address whether decreases in Puc activity rescue arm dorsal closure by upregulating JNK signaling and/or dpp expression in leading edge cells, a leading-edge-specific GAL4 driver (LE-Gal4) was used to activate either JNK or Dpp signaling in an arm mutant. Overexpression of DTak specifically in leading edge cells ameliorated the arm dorsal phenotype (Fig. 7B) while ectopic expression of a dominant-negative DTak transgene (UAS-DTakKN1-J) throughout the epidermis augmented arm's dorsal closure defects (Fig. 7C). Furthermore, expression of either dpp (Fig. 7D) or activated thick veins (tkv; Fig. 7E,F), a Dpp receptor, in leading edge cells of arm mutants partially suppressed the dorsal closure defects associated with loss of Arm function. Together, these results suggest that elevation in
JNK signaling, either via inactivation of Puc or overexpression of JNK pathway kinases, can suppress arm's dorsal defects while reductions in JNK signaling in leading edge cells augment arm's dorsal closure defects.

Constitutive activation of the Wg pathway results in the upregulation of dpp expression (Fig. 6). To assess whether activation of the Wg pathway is sufficient to suppress defects associated with a loss of JNK signaling, Wg (data not shown) or Arm$_{S10}$ (Fig. 7H) were misexpressed in the kay mutant background. Although it was previously demonstrated that activation of Dpp signaling in the dorsalmost epidermal cells rescues JNK pathway mutants (e.g., Hou et al., 1997), constitutive activation of the Wg cascade fails to promote dpp expression in leading edge cells (data not shown) or to rescue the dorsal closure defect of kay (Fig. 7G versus H). Arm's role during dorsal closure can be bypassed by activating Dpp signaling whereas the requirement for Kayak remains even if the Wg pathway is activated. Therefore, Arm may amplify JNK-dependent expression of dpp. JNK may be required for dpp-independent events during dorsal closure, or both. Finally, Arm likely contributes to dorsal closure in a Wg-independent way, through its maintenance of cadherin/catenin-based adherens junctions.

**Wg signaling is required to coordinate dorsal closure**

The dorsal closure defects of arm mutants were previously ascribed to Arm's role in adherens junctions, as wgnull mutants (Nusslein-Volhard and Wieschaus, 1980) and arm mutants specifically affecting Wg signal transduction are not completely open dorsally. However, given the similar alterations in the transcriptional program of leading edge cells caused by defects in either JNK or Wg signaling, we re-examined dorsal closure in Wg pathway mutants. wgnull cuticles have significant defects in dorsal pattern, characterized by loss of dorsal hairs and the presence of abnormal cuticular structures (Fig. 4K,L). While most wgnull mutants are closed dorsally, 18% remain open at the dorsal anterior end (Fig. 4L); similar defects are seen in embryos maternally and zygotically mutant for dsh (Fig. 4M) or arm$^{X19}$ (Fig. 4O), or in embryos.
expressing dTCFAN throughout the epidermis (Fig. 4P). Thus, the endpoint of dorsal closure, the dorsal cuticle pattern, is altered in wg-class mutants.

These analyses of cuticle pattern and dpp expression suggest that Wg signaling regulates dorsal closure. To examine this directly, the cell shape changes that accompany dorsal closure were monitored by confocal microscopy, utilizing fluorescent-phalloidin to label filamentous actin and anti-PTyr antibody to label adherens junctions (Fig. 8). In wild-type embryos, cells begin to change shape during germband retraction (Fig. 8A,J). Cells of the leading edge are organized into a single well-defined row, and elongate in the DV axis by the end of germband retraction (Fig. 8B,K). As dorsal closure initiates, an additional 3-4 lateral cell rows also begin to stretch ventrally. Leading edge cells meet first at the posterior (Fig. 8C,L). Next, anterior leading edge cells meet and the embryo completes dorsal closure.

Loss of wg completely blocks the well-ordered cell shape changes that normally accompany dorsal closure. Leading edge cells never elongate along the DV axis (Fig. 8D,M); instead they stretch along the AP axis (Fig. 8E,N). This AP stretching may correlate with the 'purse string' tightening of actin filaments thought to help drive dorsal closure (Young et al., 1993). Presumptive leading edge cells of wg mutants accumulate actin and PTyr where they contact the amnioserosa, though they do so in an uneven fashion compared to wild type (Fig. 8E,N; data not shown). In addition to these defects, certain lateral cells contract like leading edge cells, stretching along the AP axis (Fig. 8N). Thus lateral cells in wg mutants may not acquire proper DV identities.

It has been hypothesized that the concerted cell shape changes characteristic of wild-type embryos are essential for dorsal closure. Thus, we were quite surprised to observe that, despite the severe abnormality in cell shape changes in a wg mutant, dorsal closure proceeds, though in a very abnormal fashion (Fig. 8F,O). Due to incomplete germband retraction, cells still cover the dorsal side at the posterior end; this may help bring posterior cells together. Gradually most leading edge cells meet at the midline and assume a cuboidal appearance.

We also examined wg;arm; puc double mutants (Fig. 9A-D), to determine what happens to cells that normally secrete dorsal and lateral cuticle. wg; puc double mutants resemble wg single mutants until germband retraction. Several differences then become apparent in wg; puc double mutants. First, dorsal closure is never initiated. Second, lateral epidermal cells, which elongate along the AP axis, activate the puc enhancer trap, thus further resembling leading edge cells (Fig. 9A). Finally, massive cell degeneration is seen in the latero lateral dorsal epidermis of late embryos (Fig. 9B,C), likely explaining the complete absence of dorsal cuticle. The puc enhancer trap comes on strongly in the cells destined to degenerate. A small group of ventral epidermal cells survives (Fig. 9D), presumably these are the cells that secrete cuticle.

In arm mutants, certain aspects of dorsal closure are more severely affected than in wg while others proceed more normally. This may reflect the fact that arm zygotic mutants retain some Wg signaling, albeit greatly reduced, but also have reductions in cadherin-catenin function. arm mutants are quite normal through germband retraction. At this point, leading edge cells initiate cell shape changes (Fig. 8C,P), but do not do so in a coordinated fashion, producing a variety of cell shapes (Fig. 8P). A subset of more lateral cells fail to elongate (Fig. 8H,Q). Most arm embryos fail to initiate dorsal closure and leading edge cells often curl under their more lateral neighbors. At a stage when dorsal closure would finish in wild-type embryos, the amnioserosa rips away from the leading edge cells (Fig. 8L,R), with detached cells resuming a cuboidal shape. arm;puc double mutants resemble arm single mutants until the point at which dorsal closure should be complete. At this point, ectopic enhancer trap expression appears in the amnioserosa and at the anterior and posterior ends of the embryos (Fig. 9F,G), as in puc single mutants. Enhancer trap expression expands to nearly fill the dorsal and lateral epidermis of the double mutant (Fig. 9I), perhaps leading to eventual cell

![Fig. 7. Ectopic Dpp signaling rescues arm's dorsal closure defects while activation of Wg signaling fails to rescue JNK signaling mutants. Arrowheads denote the posterior most extent of the dorsal hole; arrows denote filzkörper, the posterior most structure. (A) Lateral view of arm511/1Y; (B) ventral view of arm511/1Y;LE-Gal4;UAS-DTak577. Note rescue of dorsal closure. (C) Lateral view of arm511/1Y;LE-Gal4;UAS-DRax577. The dorsal defect is enhanced. (D) Ventral view of arm511/1Y;LE-Gal4;UAS-dpp. Note rescue of dorsal closure. (E,F) Ventral and dorsal views of arm511/1Y;UAS-tkr2D11D;LE-Gal4. Note rescue of dorsal closure. (G) Dorsal view of kay2 arm-Gal4::VP16/kay2 arm-Gal4::VP16/kay2. a denotes ventral denticle belts. (H) Dorsal view of UAS-arm511/y; kay2 arm-Gal4::VP16/kay2 arm-Gal4::VP16/kay2. Note the loss of denticles.](image-url)
Fig. 8. Dorsal closure is aberrant in both wg and arm. Embryos labeled for PTyr to view cell boundaries. Dorsal closure in successively later wild-type (A-C, J-L), wg
\textsuperscript{22} (D-F, M-O) and arm\textsuperscript{XP3} (G-I, P-R) embryos. Anterior is to the left. (A,J) Lateral views, late stage 12 wild type. Leading edge cells (arrows) begin change shape and align in a row; lateral neighbors remain cuboidal (arrowhead). (B,K) Dorsal and lateral views of stage 14 wild type. Leading edge cells are elongated (arrows); lateral neighbors are beginning to change shape (arrowheads). (C,L) Late stage 14 wild type. Both leading edge (arrows) and lateral cells (arrowheads) are highly elongated. At the leading edge and where cells join they accumulate PTyr (open arrowheads). (D,M) Lateral views of stage 12-13 wg
\textsuperscript{0} mutants. Segmentation is not apparent. Germband retraction is not completed. Leading edge cells (arrows) never initiate proper cell shape changes, nor do they form a single row. During stage 13, they elongate in the AP axis, as do certain lateral cells. (E,N) Lateral views of stage 14 wg
\textsuperscript{22} mutants. Leading edge cells (arrows) and certain lateral neighbors (arrowheads) are highly elongated. Dorsal closure proceeds. (F,O) Lateral/dorsal views of stage 15 wg
\textsuperscript{22} mutants. Dorsal closure has gone to (0) or nearly to (F) completion. Upon completion of closure, leading edge cells (arrows) resume a cuboidal shape, and their more lateral neighbors remain cuboidal (arrowhead). Cells at the midline are not well-aligned nor do they accumulate high levels of PTyr (open arrowheads). (G,P) Lateral views of stage 13 arm\textsuperscript{XP3} mutants. Leading edge cells initiate elongation, but are much less uniform in shape (arrows). (H,Q) Lateral views of stage 14 arm\textsuperscript{XP3} mutants. Leading edge (arrows) and lateral cells (arrowheads) elongate in the DV axis but do so irregularly. (I,R) Lateral views of stage 14/15 arm\textsuperscript{XP3} mutants. The amnioserosa rips from the leading edge (open arrowheads). Leading edge cells attached to the amnioserosa remain elongated (arrows), but those that detach resume a cuboidal shape (arrowheads).

dead of the dorsal and lateral epidermis as in wg;\textit{puc} embryos.

DISCUSSION

Wg and JNK signaling co-operate during embryogenesis

The JNK and Wg signaling pathways were thought to function in distinct domains, with JNK regulating dorsal closure and Wg regulating segment polarity. Here we demonstrate that Wg signaling is critical for normal dorsal closure and that a negative regulator of the JNK pathway, Puc, plays an unexpected role in ventral patterning. This connection emerged from the observation that reduction in Puc function suppresses both the dorsal closure and ventral segment polarity phenotypes of non-null mutations in the Wg pathway.

Puc encodes a MAPK phosphatase that antagonizes JNK signaling (Martin-Blanco et al., 1998). Thus the simplest hypothesis to explain our results is that puc suppresses arm by hyperactivating the JNK pathway. Consistent with this, the puc enhancer trap, a JNK target gene, is ectopically activated in DISCUSSION crosstalk occurs:
both DMKK4 and Hep can activate Bsk, while *Drosophila* p38 orthologs can phosphorylate Djun and ATF2, both known targets of Bsk (Han et al., 1998). Thus, the JNK signaling pathway may function redundantly with other MAPK pathways, both in planar polarity and in segment polarity. As JNK-independent expression of *puc* has also reported (Zecchini et al., 1999), additional studies will be required to assess the ability of Puc to antagonize other MAPK signaling pathways. While these circumstantial arguments are consistent with a role for the JNK pathway in ventral patterning, the caveats raised by the lack of effects of loss-of-function JNK mutations leave open the possibility that Puc has a role in ventral patterning that is independent of its role in regulating JNK activity – for example, it could directly regulate the canonical Wg pathway.

**Wg and JNK – one pathway or two?**

Our data, combined with previous studies of JNK signaling (reviewed in Noselli and Agnes, 1999), further suggest that Wg and JNK signaling act in parallel during dorsal closure. Both pathways regulate *dpp* expression in dorsal epidermal cells and are required for the proper coordinated cell shape changes to occur. These data are compatible with several different models. It may be that the two pathways both impinge on the same process and the same target gene, but that they do so in response to independent upstream inputs. However, when our data is combined with other recent studies, a potential direct connection between the Wg and JNK pathways is suggested. Using both genetics and in vitro studies, others demonstrated that JNK pathway kinases act downstream of Frizzled and Dsh in planar polarity and that Dsh can activate the JNK signaling cascade directly (Boutros et al., 1998; Li et al., 1999). This suggested that Dsh may function as a binary switch, deciding between the canonical Wg pathway and the JNK pathway during the establishment of segment polarity and planar polarity, respectively. Here, we demonstrate that both the canonical Wg and the JNK pathways are required for proper dorsal closure, and that both pathways affect expression of the same target gene, *dpp*. One plausible model accommodating these data is that Wg, acting via Frizzled receptors and Dsh, activates both the JNK pathway and the canonical Wg pathway simultaneously and in parallel during both dorsal closure and ventral patterning. The possibility that Wg activates both pathways, while exciting in principle, remains quite speculative, and must now be tested by more direct biochemical and cell biological means.

**Wg as a permissive signal**

It also is possible that Wg functions as a permissive signal required to allow other effectors to promote *dpp* expression. For example, *dTcf* could repress *dpp* expression in the absence of Wg signaling by recruiting Groucho (Cavalleri et al., 1998), a transcriptional repressor, to the *dpp* promoter. Wg signaling might relieve this repression by displacing Groucho with stabilized Arm. Consistent with this hypothesis, constitutive activation of Arm fails to rescue the dorsal closure defects of *key* mutants. Thus activation of the canonical Wg signaling pathway is necessary but not sufficient to promote *dpp* expression. Wg signaling may thus only amplify JNK-dependent expression of *dpp* in the dorsal epidermis.

One possible intersection between MAPK signaling cascades and TCF-mediated repression has been reported (reviewed in Bowerman and Shelton, 1999). Transcriptional repression of Wnt target genes in *C. elegans* depends upon POP-1, a TCF family member. POP-1 repressor activity is regulated by Mom-4, a TAK1-like kinase, and Lit-1, a Nemo-like MAP kinase relative (Nlk). In mammalian cells, the
transcriptional activity and DNA-binding properties of TCF can be repressed by Tak1/JNK activation. Therefore, the canonical Wg and MAPK/JNK pathways might converge at dTCF, with MAPK kinase signaling affecting dTCF activity. Additional studies will be required to assess the mechanism by which these pathways interact.

Cell shape changes and Dpp appear dispensable for dorsal closure

The current model suggests that a sequential series of cellular events drive dorsal closure. Leading edge cells are thought to initiate closure by elongating in the DV axis and upregulating Dpp, thus signaling lateral cells to initiate similar cell shape changes. Loss of Wg or Dpp expression in leading edge cells is lost in wg mutants; however, the lateral epidermal sheets usually meet at the dorsal midline. In contrast, while cell shape changes are initiated in arm mutants, other than the JNK pathway likely depends upon activation by signals other than Wg and must affect other processes in addition to Dpp signaling. Further work is required to clarify the semi-redundant mechanisms regulating dorsal closure.

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REFERENCES


A Screen for Mutations That Suppress the Phenotype of *Drosophila armadillo*, the β-Catenin Homolog

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ABSTRACT

During development signaling pathways coordinate cell fates and regulate the choice between cell survival or programmed cell death. The well-conserved Wingless/Wnt pathway is required for many developmental decisions in all animals. One transducer of the Wingless/Wnt signal is Armadillo/β-catenin. Drosophila Armadillo not only transduces Wingless signal, but also acts in cell-cell adhesion via its role in the epithelial adherens junction. While many components of both the Wingless/Wnt signaling pathway and adherens junctions are known, both processes are complex, suggesting that unknown components influence signaling and junctions. We carried out a genetic modifier screen to identify some of these components by screening for mutations that can suppress the *armadillo* mutant phenotype. We identified 12 regions of the genome that have this property. From these regions and from additional candidate genes tested we identified four genes that suppress *armadillo*: *dTCF*, *puckered*, *head involution defective* (*hid*), and *Dpresenilin*. We further investigated the interaction with *hid*, a known regulator of programmed cell death. Our data suggest that Wg signaling modulates Hid activity and that Hid regulates programmed cell death in a dose-sensitive fashion.

The development of a fertilized egg into a multicellular organism requires coordination of many processes. Each cell must choose the proper cell fate and must also assume its place as part of an organized tissue. In addition, apoptosis (programmed cell death; PCD) plays an important role in shaping an organism by eliminating unneeded cells. One conserved pathway that directs cell fate decisions in many animals is the Wingless (Wg)/Wnt signal transduction pathway (proteins listed as X/Y represent nomenclature in Drosophila/mammals). Loss-of-function mutations in this pathway are lethal, while inappropriate activation can be oncogenic. Wg/Wnt signals are transduced by homologous components in Drosophila, Xenopus, and mammals (reviewed in *Polakis* 1999). During normal development, most cells do not receive Wg/Wnt signals. In these cells the pathway is kept off through the actions of several proteins, including Zeste/white3/GSK3β, the tumor-suppressor adenomatous polyposis coli, and axin, which work in conjunction to target Armadillo (Arm)/β-catenin (βcat) for degradation via the proteasome. Arm/βcat is thus the pivotal component in the pathway. When Wg/Wnt is absent, cytoplasmic levels of Arm/βcat are very low. However, Wg/Wnt signal relieves the destruction of Arm/βcat. Arm/βcat accumulates, translocates into the nucleus, and binds dTCF/TCF, forming a bipartite transcription factor that turns on Wg/Wnt-responsive genes.

The components of the Wg pathway are encoded by a subset of the segment polarity genes, mutations that affect cell fate in the embryonic epidermis. In normal fly embryos, anterior cells of each segment secrete denticles, while posterior cells secrete naked cuticle. Wg signal directs cells to choose posterior fates and thus secrete naked cuticle. In an embryo mutant for *wg* or other positively acting components of the Wg pathway, cell fates are altered such that all surviving cells secrete denticles. It is important to note, however, that in a *wg* mutant many epidermal cells fail to survive to secrete cuticle, instead undergoing PCD. Embryos mutant for genes in either the Wg or the Hedgehog pathways have elevated levels of epidermal PCD (*Martinez Arias* 1985; *Klingensmith et al.* 1989; *Pazdera et al.* 1998).

Arm's role in Wg signaling is not its only function. The earliest requirement for Arm is in cell adhesion (*Cox et al.* 1996). Arm/βcat is an essential component of epithelial cell-cell adherens junctions (reviewed in *Provost* and *Rimm* 1999). The core components of this junction are classic cadherins, transmembrane proteins that mediate homotypic adhesion between neighboring cells. Arm/βcat binds to the cadherin cytoplasmic tail. α-Catenin then binds to Arm/βcat, linking the actin cytoskeleton to adherens junctions. In Drosophila, Arm helps assemble adherens junctions very early during embryogenesis. This is initiated by maternal Arm, which is supplemented by zygotic Arm once transcription begins. If the embryo lacks maternal and zygotic Arm, it

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does not form proper adherens junctions, and cells of the cellularized blastoderm cannot form epithelia (Cox et al. 1996). In addition to the essential role that Arm/βcat and adherens junctions play in embryogenesis, loss-of-function mutations in the cadherin-catenin system contribute to tumorigenicity, as tumor cells must alter their adhesive properties to metastasize.

While the roles of Arm/βcat in Wg/Wnt signaling and adherens junctions have become clearer, many questions remain concerning both processes. In addition, biochemical approaches identified many other proteins that bind βcat, perhaps implicating it in other functions; for example, Arm/βcat binds the epidermal growth factor (EGF) receptor at the cell surface (Hoschuetzky et al. 1994), the actin-binding protein fascin in the cortex (Tao et al. 1996), Presenilin proteins, presumably in the endoplasmic reticulum (ER) (Zhou et al. 1997; Yu et al. 1998), and the transcription factor Teashirt (Galley et al. 1998). One strategy to identify novel proteins involved in cell adhesion and Wg signaling and simultaneously to search for biological functions of the interaction of Arm with other partners is to look for mutations that interact genetically with arm.

In designing such a genetic screen, we took advantage of Arm’s dual roles in signaling and adhesion. It has been suggested that cells may use this coupling, allowing one process to regulate the other via competition for a limited pool of Arm. Although in wild-type Drosophila embryos more than enough Arm is synthesized to fulfill its roles in both signaling and adhesion, one can manipulate the pool of Arm to make signaling and adhesion competitive. For example, if one expresses excess cadherin, it titrates out all the Arm, leaving none available for Wg signaling and resulting in a segment polarity phenotype (Sanson et al. 1996). We utilized this balance between Arm assembled into adherens junctions and that remaining for Wg signaling to create a sensitized genetic background. We reduced the amount of available Arm until adhesion and Wg signaling became competitive by using a zygotic arm mutant that retains wild-type maternal Arm, sufficing for Arm’s role in adherens junctions (Cox et al. 1996). With most wild-type maternal Arm assembled in adherens junctions, the embryo drops below the critical threshold of Arm necessary for Wg signaling, resulting in segment polarity defects. Such an embryo is very sensitive to slight changes in arm dose; for example, doubling the maternal Arm substantially suppresses the segment polarity phenotype (Wieschaus and Noell 1986). Thus this represents a sensitized background well suited for a modifier screen. Mutations in genes that affect adherens junction assembly, which negatively regulate Wg signaling or encode other proteins that bind the limited supply of maternal Arm, could all potentially suppress the segment polarity phenotype of arm. We previously demonstrated the feasibility of this idea, showing that reduction in DE-cadherin suppressed arm’s segment polarity phenotype (Cox et al. 1996).

We used the sensitized background of a zygotic arm mutant to carry out a modifier screen, looking for changes in the segment polarity phenotype. We screened through deficiencies covering >80% of the second, third, and fourth chromosomes, searching for regions of the genome containing a gene or genes that, when heterozygous deficient, suppress the cuticle phenotype of arm. We found 12 such regions and identified four genes with this property. One interactor is the PCD-promoting gene head involution defective (hid). Our data suggest that Hid acts as a dose-sensitive regulator of PCD in the ventral epidermis of segment polarity mutants.

MATERIALS AND METHODS

Fly stocks: References for mutants used were the following: armY97, armX1, and zw3/warm (Cox et al. 1996; Peifer et al. 1994); hid (Grether et al. 1995); hid (Arnett and Lengyel 1991); Df(3)H99 (White et al. 1994); yw (Nüsslein-Volhard and Wieschaus 1980); UAS-p35 (Hay et al. 1994); other mutations, http://flybase.bio.indiana.edu/. The deficiency kits were from the Bloomington Drosophila Stock Center, the Pletschs from Bloomington or the Berkeley Drosophila Genome Project (BDGP), and the Dprensinill alleles from D. Curtis.

Cuticle preparations and counting: Cuticle preparations were as in Wieschaus and Nüsslein-Volhard (1986). Care was taken to be consistent in cuticle preparations, as differences in baking and pressing alter cuticle appearance. If the first cross suggested an interaction, the cross was repeated. Each candidate interacting region was tested in two or more separate crosses, with ≥200 cuticles scored per cross. Percentage of suppression equaled the number of cuticles in the least severe classes divided by the total number of cuticles scored.

Terminal transferase dUTP nick end labeling (TUNEL), phalloidin and antibody staining: TUNEL was done using reagents from Boehringer Mannheim (Indianapolis). Embryos were dechorionated in 50% bleach, fixed in 1:1 4% formaldehyde:heptane for 30 min, hand devitellinized, rinsed once in TdT reaction buffer (2.5 mM CoCl2, 1× transferase buffer), and reacted in TdT reaction mix (50 units terminal transferase, 2.1 10 μM final concentration of dUTP:dUTP-biotin in reaction buffer) for 3 hr at 37°C. After washing three times for 10 min in PBS + 0.1% Triton X-100 (PBT), the end-labeling was first amplified using the Vectastain kit (Vector Labs, Burlingame, CA) as recommended by the manufacturer, amplified with Cy3-Streptavidin (New England Nuclear, Boston), and washed three times for 10 min in PBT. BODIPY-phalloidin (Molecular Probes, Eugene, OR) was added during the avidin-biotin reaction of the first amplification. Antiphosphohistone labeling was as in Cox et al. (1996).

Phosphohistone H3 staining: The 2- to 7-hr-old embryos were dechorionated in 50% bleach, fixed in 1:1 5% formaldehyde:heptane for 20 min, blocked (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP-40, 5 mg/ml BSA) at 4°C for 2 hr and stained overnight at 4°C with 1:1000 antiphosphohistone H3 (Upstate Biotechnology, Lake Placid, NY) and 1:500 anti-β-gal (Boehringer Mannheim, Indianapolis). Secondary antibodies were from Molecular Probes. Pictures of the ventral epidermis and dorsal germband were taken, mitotic figures (stained for phosphohistone H3) counted, and means and standard deviations calculated.
in genetic screens modifiers are also found that operate by unexpected mechanisms.

The feasibility of this hypothesis was supported by two observations. We previously found that heterozygosity for a chromosomal deficiency removing DE-cadherin, Df(3R)E2, suppresses the embryonic phenotype of arm\(^{593}\)—the cuticle is longer, the dorsal closure defect is substantially reduced, and denticle diversity is partially restored (Figure 1, B vs. C; Cox et al. 1996). We presume that reducing the gene dose of DE-cadherin by half creates an embryo with fewer Arm/Cadherin complexes. Although this has no apparent effect on cell-cell adhesion, wild-type maternal Arm is freed up to function in Wg signaling, leading to a suppressed phenotype. A similar suppression of arm was seen by removing the zygotic contribution of one of Arm’s negative regulators, Zw3 (Figure 1, D vs. E; we tested this on arm\(^{5119}\), a less severe allele).

A modifier screen for Arm interactors: These examples demonstrated that a 50% reduction in the dose of certain genes suppresses arm. We thus screened for dose-sensitive modifiers. Rather than examining single genes one by one by mutagenesis, we evaluated large regions of the genome simultaneously by making animals heterozygous for chromosomal deficiencies that remove many genes. We obtained the “deficiency kits” for three of the four chromosomes from the Bloomington Drosophila Stock Center. These kits are designed to delete as much of the chromosome as possible using the fewest stocks; 70–80% of the euchromatin was covered by this collection of Deficiencies when we obtained them. We extended our analysis by obtaining additional Deficiencies that either covered regions not covered in the kit or overlapped interacting Deficiencies. We estimate we covered >80% of the autosomes. We have not examined the X chromosome thus far, as arm is on the X and the screen would require recombination of arm onto each deficiency. To carry out the screen, we crossed virgin arm\(^{593}\) females to males heterozygous for each deficiency and prepared cuticles from the dead embryonic progeny (Figure 2A). One-quarter of the progeny are arm\(^{593}\)/Y, a somewhat weaker allele. (E) arm\(^{5119}\) zw3\(^{921}\)/Y. When zygotic zw3 is removed (leaving only maternal zw3), the phenotype of arm\(^{5119}\) is suppressed.

RESULTS

Strategy for the screen for modifiers: arm\(^{593}\) encodes a carboxy-terminally truncated Arm protein that cannot function in Wg signaling and has almost no function in adherens junctions (Cox et al. 1996). In an arm\(^{593}\) zygotic mutant, maternal wild-type Arm provides sufficient function for adherens junctions. However, as nearly all maternal Arm is recruited into junctions (Cox et al. 1996), little Arm remains to transduce Wg signal, resulting in a strong segment polarity phenotype (Figure 1, A vs. B). We reasoned that if one elevated the level or function of the limiting pool of maternal Arm, this should suppress the defect in Wg signaling. We hypothesized that this could occur by freeing maternal Arm from junctions, reducing the effectiveness of negative regulation of Arm’s role in Wg signaling by reducing the level of a distinct Arm-binding protein. Of course
low frequency (0.5%), \(arm^{XP33}/Y\) cuticles have the least severe phenotype: these are nearly wild type in length, have greater denticle diversity, and are dorsally closed (they retain an anterior hole). If one does a similar analysis of \(arm^{XP33}/Y; Df-DE-cadherin/+\) embryos, as an example of suppression, one finds that the phenotypic distribution is strongly shifted toward the less severe end (Figure 2B)—in this example, 33% of the cuticles fall in the least severe classes (embryos to the left of the dotted line in Figure 2B). On the basis of this, we focused on the frequency of embryos in the least severe classes. To score whether a Deficiency suppressed the \(arm^{XP33}\) phenotype, we prepared cuticles from the dead embryos, scored their phenotypes, and calculated the percentage of cuticles in the least severe classes; if this was at least six times the frequency in the control (i.e., \(\geq 3\%\)), we scored this as an interaction.

By these criteria, 32 deficiencies interacted with \(arm^{XP33}\) (Table 1); a representative suppressed cuticle is shown in Figure 3B. Tables 1 and 2, Figure 4, and APPENDICES A and B summarize the screen, showing which regions were covered by deficiencies and which regions interacted. In all cases the suppression was qualitatively similar; embryos in the least severe class showed an increase in cuticle length, improvement in dorsal closure, and an increase in denticle diversity. The fraction of cuticles in the least severe phenotypic class ranged from 3 to 40\% (each number is an average of two to three independent crosses; Tables 1 and 2). We retested each interacting stock—all reliably interacted although in some cases the percentage of suppression varied. Of the 32 stocks that interacted, we arbitrarily made a cutoff between “weak” and “strong” interactions at the level of 6\% of the embryos in the least severe phenotypic classes. Eighteen Deficiencies were thus classified as weak interactors, with 3–5.9\% of the cuticles in the least severe category (Table 1; APPENDICES A and B). Although this degree of suppression was reproducible, there were enough regions that suppressed \(arm^{XP33}\) more robustly that weakly interacting regions were not investigated further. We noted in passing that six stocks had hemizygous dominant cuticle phenotypes other than effects on segment polarity (Table 2B); one was also one of the strong interactors.

**TABLE 1**

Summary of the Deficiency screen by chromosome

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>No. of stocks/chrom.</th>
<th>(0–2.9%)</th>
<th>(3–5.9%)</th>
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</table>
ment polarity defects. The wild-type maternal contribution of Arm appears to completely provide adherens junction function, so reducing levels of components required for adherens junction function by 50% apparently does not affect epithelial integrity in arm mutant. In fact, when Müller and Wieschaus examined embryos homozygous for large deficiencies, they found no regions that were zygotically essential for adherens junction assembly and few that had a strong effect on junction function (MÜLLER and WIESCHAUS 1996). We realized in retrospect that the severity of the arm segment polarity phenotype made it unlikely one could reliably recognize an enhancer of this defect.

One possible confounding factor was that mutations on the Balancer chromosomes with which the Deficiency chromosomes were heterozygous could have been the true cause of the phenotypic suppression. We think this is quite unlikely, as only a small number of Balancer chromosomes were used and none showed a consistent effect on the arm phenotype. A second potential problem is that second site mutations on the Deficiency chromosomes could in principle be responsible for certain observed interactions. This is highly unlikely for the seven strongly interacting regions that are defined by either two or more interacting Deficiencies or by a Deficiency and an identified gene (Figure 4). For the other five strongly interacting regions, some may be due to linked mutations outside the Deficiency interval, although given the overall frequency at which interactions were detected, we think this is unlikely to be the case for all.

Finding interactors by testing candidate genes: Our first approach to identify the gene(s) within each Deficiency responsible for the interaction was to test candidate genes in each region. We considered as candidate genes those with a mutant phenotype indicating an effect on cell fate choice in the ventral epidermis, genes known to act in Wg signaling, and genes known to affect cell-cell junctions or the actin cytoskeleton. We identified one interactor by this candidate gene approach and ruled out many other candidates by two methods: testing complementation between a candidate and the Deficiency responsible for the interaction (Figure 4). For the other five strongly interacting regions, some may be due to linked mutations outside the Deficiency interval, although given the overall frequency at which interactions were detected, we think this is unlikely to be the case for all.

We tested four candidate genes that are part of the Wg signaling transduction pathway or that affect segment polarity: dTCF, cubitus interruptus, naked, and sog. Removing one copy of the fourth chromosome gave a very strong interaction. In examining candidates on the fourth chromosome, we found that mutations in the gene encoding the DNA-binding protein dTCF, which is required for Wg signaling, suppress arm. This was a surprise and revealed a previously unexpected role for dTCF as a repressor as well as an activator of Wg-responsive genes (Cavaillé et al. 1998). However, while null alleles of dTCF interact strongly, they do not suppress arm to the same degree as removing the entire...
### TABLE 2

Deficiencies that had a strong interaction with *arm*<sup>933</sup>

<table>
<thead>
<tr>
<th>A. Original interacting deficiency</th>
<th>Smallest region</th>
<th>% Supp.</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(2L)spad, a[1] dp[ox1]/CyO</td>
<td>28B3-4:28C</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>In(1)w[md4h], y[1]; Df(2L) TE29Aa-11/CyO</td>
<td>28E4-7:29B2-C1</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>Df(2L)TWI37, co[1] bw[1]/CyO, Dp(2;2) M(2) m[+]</td>
<td>36CD1-E1; 36E1-F2</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>Df(2R)ST1, pr[1] cn[*]/CyO</td>
<td>42B3-5:42E</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Df(2R)PC4/CyO</td>
<td>55C;55F</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>Df(2R)017/SM1</td>
<td>56F5;56F15</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>Df(3L)Cat, ri[<em>] e[</em>]/TM6</td>
<td>75B8-75F1</td>
<td>9.8</td>
<td>hid</td>
</tr>
<tr>
<td>Df(3R)p712, red[1] c[1]/TM3</td>
<td>84D3-5:84F1-2</td>
<td>10.0</td>
<td>puc</td>
</tr>
<tr>
<td>C(4)RM, ci[1] cy[R]</td>
<td>101F1;102B</td>
<td>37.0</td>
<td>dTCF</td>
</tr>
<tr>
<td>B. Hemizygous dominant phenotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dp(3;1)2-2, w[1118] z; Df(3R)2-2/TM5</td>
<td>81F;82F10-13D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Df(3R)p712, red[1] c[1]/TM3</td>
<td>84D4-6:85B6,</td>
<td>25D85B6</td>
<td></td>
</tr>
<tr>
<td>Df(3R)23D1, cy[306]/TM3 Sb[1]/mus309[Horka] e[1]</td>
<td>93E-94F1-6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A: Column 1, the original interacting Deficiencies from the Deficiency kits; column 2, the smallest interacting region, derived from comparing interacting and noninteracting Deficiencies (see APPENDIXES A and B for details); column 3, the percent of individuals in the weakest phenotypic classes; column 4, identified interacting genes. B: Six Deficiencies were associated with partially penetrant dominant phenotypes. One also was a suppressor.

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**Strongly interacting Deficiencies**

<table>
<thead>
<tr>
<th>Strongly interacting Deficiencies</th>
<th>Confirmed by z = 2 independent interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2L</td>
<td>Defined by single interacting Deficiency</td>
</tr>
<tr>
<td>One region or two?</td>
<td></td>
</tr>
<tr>
<td>2R</td>
<td></td>
</tr>
<tr>
<td>3L</td>
<td></td>
</tr>
<tr>
<td>hid</td>
<td></td>
</tr>
<tr>
<td>3R</td>
<td>dTCF</td>
</tr>
</tbody>
</table>

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**Figure 4.**—Schematic summary of the screen. We estimate that ≥80% of the euchromatin of chromosomes 2, 3, and 4 were covered. Regions covered are represented by the black portions of the chromosomes; white portions are regions for which we were unable to find deficiencies. Black boxes below chromosomes represent regions containing putative suppressor(s) defined by the overlap between two or more deficiencies, or where an interacting gene was defined. White boxes represent regions defined by a single interacting Deficiency (see APPENDIX for details).
it complemented this Deficiency and was thus ruled out. Two deficiencies, Df(2L)TE29 and Df(2L)sd729, are in the vicinity of wg. While wg is a positively acting component of the pathway, our experience with DTCF made us cautious in ruling it out without a test. We found that: (1) wg complements Df(2L)TE29 and (2) a wg null does not suppress arm. This ruled out wg, although Dllnt4, which maps near wg (Graba et al. 1995), remains a candidate. Finally, we tested alleles of two segment polarity genes that fell outside regions included in the Deficiencies in the kit: hedgehog and teashirt, which encodes a transcription factor that physically and functionally interacts with Arm (Gallet et al. 1998). Neither suppressed arm<sup>993</sup>.

We also tested several genes with roles in cell-cell adhesion or cytoskeletal function. One was DE-cadherin (shotgun), which we already knew could suppress arm. Df(2)017 was suggested by its cytology to remove DE-cadherin, but both an allele of DE-cadherin and the small deficiency Df(2)E2 that removes DE-cadherin (Uemura et al. 1996) complement Df(2)017. Thus this interaction is due to a different gene. Three other genes that regulate the cytoskeleton, enabled (en), Gerlet et al. 1995), quail (Mahajan-Miklos and Cooley 1994), and scraps (Schupbach and Wieschaus 1989), map to regions covered by interacting Deficiencies (56B5, 36C2-11, and 43E7, respectively). ena is an actin cytoskeleton regulator, quail encodes a vinculin-like protein thought to associate with actin, and scraps is required for the cytoskeletal events of cellularization. ena was included in interacting deficiency Df(2R)PC4 by complementation (we did not test quail and scraps by complementation). However, when we tested alleles of all three genes, none suppressed arm<sup>993</sup>. 18-wheeler, a putative cell-adhesion molecule (Eldon et al. 1994) that maps in or near Df(2R)017, also did not suppress arm<sup>993</sup>.

As a partial test of the effectiveness and completeness of the screen, we also tested a series of additional candidate genes, some of which fell outside Deficiencies in the kit and others of which were probably included in these Deficiencies but which we expected might physically or functionally interact with Arm. The vast majority did not show an interaction. We tested a variety of genes encoding components of other signal transduction pathways that pattern the dorsal or ventral epidermis: (1) the Hedgehog pathway, hedgehog<sup>2</sup> and Dpp pathway, decapentaplegic<sup>2</sup> and screw<sup>5</sup>; (3) the EGF receptor (EGFR) and other receptor tyrosine kinase pathways, spitz<sup>214</sup>, vein<sup>122</sup>, argos<sup>97</sup>, Egfr<sup>18</sup>, ras83D<sup>18</sup>, rold<sup>18</sup>, yan<sup>50,12</sup>, and torso<sup>1</sup>; and (4) the Jun N-terminal kinase pathway, basket and Djun<sup>1</sup>. Of these, only spitz<sup>214</sup> interacted, and even in this case, only 3.8% fell into the weakest phenotypic categories, just above our cutoff for a weak interaction. We also tested five genes affecting the cytoskeleton or cuticle integrity: krotzkopf verkehr<sup>1</sup>, myoblast city<sup>3</sup>, shroud<sup>1</sup>, steamer duck<sup>10,11</sup>, and scraps<sup>5</sup>. None interacted. Finally, we tested one candidate among proteins that interact with mammalian β-catenin but for which the function of this interaction is not known. This was Drosophila presenilin, homolog of the mammalian presenilin family of transmembrane proteins (reviewed in Haass and De Strooper 1999). Mammalian Presenilins bind mammalian β-catenin (Zhou et al. 1997; Murayama et al. 1998; Yu et al. 1998; Levesque et al. 1999). Further, misexpression and other experiments suggest that mammalian Presenilins may regulate Wnt signaling (Murayama et al. 1998; Zhang et al. 1998; Kang et al. 1999; Nishimura et al. 1999a). In contrast to the other candidates tested, D. presenilin showed a very strong interaction. Heterozygosity for D-pesenilin strongly suppressed arm<sup>993</sup> (14.6% with weakest phenotypes; Figure 3, A vs. C) and also suppressed the zygotic null arm allele, arm<sup>1015</sup> (Figure 3, E vs. F). A surprise from these results was that although D-pesenilin was removed by two of the Deficiencies tested, Df(3L)ri-79c and Df(3L)rdgC-co2, neither showed a significant interaction (percentage of suppressions = 1.8 and 1.5%, respectively). This suggests that some interactions are sensitive to genetic background, and thus not all potential haplo-insufficient interactors were identified in our screen.

**P-element lethals that interacted:** Our second approach to identifying genes responsible for an interaction was to use the collection of P-element-induced lethal mutations (hereafter called P-lethals) characterized by the Berkeley Drosophila Genome Project. These lethals are caused by P-element transposon insertions and are thus molecularly tagged, facilitating cloning. The available P-lethals are estimated to hit ~25% of essential genes (Spradling et al. 1999). One caveat to using these mutations to uncover a dose-sensitive suppressor is that there is no guarantee that the P-lethal will be a null allele, as is a Deficiency. P transposons tend to insert either in the 5' untranslated region or in introns, thus creating mutations that often are not null in phenotype and thus do not, when heterozygous, reduce gene function by 50%.

We obtained the P-lethals available from the Bloomington Stock Center (81 stocks) and the Kiss collection (73 stocks) in each of the interacting regions and tested their ability to suppress arm<sup>993</sup>. A list of the P-lethals tested is in a data supplement at http://www genetics.org/cgi/content/full/155/4/1725/DC1. Of the P-lethals tested, we found two that suppressed arm<sup>993</sup>. One of these, 1(3)A251.1, mapped to region 84E. Of these, by examination of its homozygous phenotype and subsequent complementation tests, we learned that this is an allele of puckered (Martin-Blanco et al. 1998). A detailed examination of the biology underlying this interaction is presented in McEwen et al. (2000).

**The apoptosis-promoting gene head involution defective is a dose-sensitive suppressor of arm.** The second P-lethal that interacted with arm was 1(3)E5014, which maps to 75C1-2 and gave as strong a suppression as either of the interacting deficiencies in this region.
A IBC, Abrams 1999). This work was initiated by a screen for genomic regions required for PCD (White et al. 1994). When chromosomal region 75C1-2 is deleted, in embryos homozygous for the small deficiency Df(3)H99, essentially all PCD in the embryo is eliminated (White et al. 1994). Subsequent analysis revealed that this chromosomal region contains three genes involved in PCD: hid, reaper, and grim (reviewed in Abrams 1999). Ectopic expression of any of these will trigger PCD. However, loss-of-function mutations are only available for hid. In hid mutants, a subset of the cells that normally undergo PCD do not do so (Gretther et al. 1995), resulting in defects in head development. In embryos homozygous for Df(3L)H99, which thus lack hid, reaper, and grim, all PCD is abolished (White et al. 1994); these embryos have slightly stronger defects in head development (Figure 5C).

hid plays an important role in PCD. Ectopic expression of hid is sufficient to induce PCD in the eye, and this is completely suppressed by the baculovirus caspase inhibitor p35, suggesting hid acts upstream of caspases (Gretther et al. 1995). hid has no clear homologs in other organisms; however, Hid overexpression triggers PCD in mammalian cells. Hid, Reaper, and Grim all share a short region of weak sequence similarity near their N termini. In Hid, this region is required for initiating cell death in mammalian tissue culture, while Hid’s C terminus is required for localization to mitochondria, an organelle involved in PCD (Haining et al. 1999).

Recent work supports the idea that Hid functions by blocking interaction between Inhibitor-of-apoptosis (IAP) family caspase inhibitors and caspases (Vucic et al. 1998; Wang et al. 1999).

Heterozygosity for hid5041 suppresses arm1(1'2) (Figure 5, D vs. E), as well as the zygotic null allele arm1(1'2) (data not shown). Heterozygosity for an X-ray-induced loss-of-function allele, hid5051, causes the same degree of suppression, further supporting the idea that hid is the gene responsible for the interaction. In addition, we generated revertants of the P element in hid5041 by mobilizing the P element and screening for viable stocks that lost the genetic marker carried by the P element. These revertant chromosomes fail to suppress arm1(1'2) (data not shown). Further reducing hid levels by making embryos homozygous for hid5041 does not increase the degree of suppression of arm1(1'2). Likewise, either heterozygosity or homozygosity for the small deficiency Df(3L)H99, which removes hid, grim, and reaper, suppresses arm1(1'2) to the same degree as removal of one copy of hid.

The suppression by hid can be mimicked by blocking PCD: PCD is elevated in segment polarity mutants (Martinez Arias 1985; Klingensmith et al. 1989; Pazderka et al. 1998). The dramatically shortened cuticle secreted by an arm mutant is presumably caused, at least in part, by loss of ventral epidermal cells via PCD. The suppression of arm1(1'2) by hid5041 could thus be due to Hid’s role in PCD; alternately, it could be due to an unknown

![Figure 5](image)

Figure 5.—Decreasing cell death suppresses arm. Wild type (A), hid1041 homozygotes (B), and Df(3L)H99 homozygotes (C) have very similar cuticle phenotypes. hid5041 and Df(3L)H99 homozygotes have head defects, but their segment polarity is normal. (D) arm+/Y (E) arm1(1'2)/Y; hid5041/+. Removing one copy of hid suppresses arm1(1'2). (F) arm+/Y; armGAL4/UAS-p35. Expression of the baculovirus antiapoptotic protein p35 in an arm1(1'2) mutant background also suppresses arm.

L(3)05014 is an allele of head involution defective (Gretther et al. 1995). This allele is likely to be a null, as the P element is inserted early in the protein-coding region. Null mutations in hid are embryonic lethal with defects in head involution during embryonic development (Abbott and Lengyel 1991; Figure 5, A vs. B), although occasional escapers survive to adulthood. More recently, it was revealed that hid mutations affect programmed cell death.

The machinery that triggers PCD in Drosophila has been the subject of intense investigation (reviewed in...
function of Hid. To test if the arm suppression results from an effect on PCD, we reduced embryonic PCD by expressing the baculovirus antiapoptotic protein p35, which acts as a caspase inhibitor; p35 suppresses the PCD triggered by hid overexpression in the fly eye (Grether et al. 1995). We found that arm<sup>222</sup> mutant embryos in which we ubiquitously expressed p35 using the GAL4-UAS system (Brand and Perrimon 1993) had a suppressed phenotype (Figure 5, F vs. D). The suppression by p35 was similar in degree to that resulting from hid heterozygosity (Figure 5E). This suggests that decreased PCD in the embryo can suppress arm and supported the idea that the interaction between hid and arm was due to hid's role in PCD.

**hid suppresses wg in a highly dose-sensitive fashion:** We next tested whether the effect was arm specific or whether reduction in PCD would suppress the phenotype caused by other reductions in Wg signaling. To do so, we examined whether reduction in PCD suppressed a null allele of wg, wg<sup>222</sup>. wg<sup>222</sup> mutant cuticles have a lawn of uniform, large denticles covering the ventral segment polarity defect (Nüsslein-Volhard and Wieschaus 1980; Figure 6A), and they are much smaller than wild type, but unlike arm mutant cuticles they are usually closed dorsally. We tested whether heterozygosity or homozygosity for either hid or for Df(3L)H99 suppressed wg<sup>222</sup>.

hid modified wg<sup>222</sup> in a dose-sensitive fashion, but the nature of the phenotypic modification was different from that seen with arm. There was not any pronounced improvement in the wg segment polarity defect; in wg<sup>222</sup>, hid<sup>9</sup> (Figure 6B) or wg<sup>222</sup>; Df(3L)H99 (Figure 6C) double mutants, all cells still secrete a uniform lawn of denticles, and the cuticle of the wg<sup>222</sup>; Df(3L)H99 double mutant remains much smaller than that secreted by a wild-type embryo, contrasting with the increase in cuticle size in arm; Df(3L)H99 double mutants. However, we found a striking effect of hid dose on the number and size of the denticles on the ventral epidermis. The number of denticles is more than doubled in wg<sup>222</sup>, Df(3L)H99 compared to wg<sup>222</sup> alone, and the denticles secreted by the double mutant are much smaller than those in the wg null (Figure 6, A vs. B and C; the change in denticle size may be less meaningful, as denticle size is also somewhat reduced in Df(3L)H99 homozygotes that are wild type for arm and wg; Figure 5C).

wg<sup>222</sup> is less sensitive to reduction in hid dose than arm, and thus the effect on wg<sup>222</sup> is additive. Removal of one copy of hid in a wg<sup>222</sup> background has only a subtle effect on cuticle pattern (data not shown), while removal of both copies of hid has a stronger effect (Figure 6, B vs. A). Removing one copy of the region covered by Df(3L)H99 has a greater effect than removing both copies of hid (data not shown), suggesting that removing all three cell death genes results in a more pronounced interaction. The effect on cuticle pattern is thus most pronounced in wg<sup>222</sup>; Df(3L)H99 double mutants (Figure 6C), which have many more, much smaller denticles than does a wg<sup>222</sup> single mutant (Figure 6A). To confirm this, we labeled embryos with phalloidin to visualize the filamentous actin in denticles and with TUNEL to identify embryos with cells undergoing PCD. Embryos homozygous for Df(3L)H99 do not label with TUNEL, as they have no PCD, allowing us to unambiguously identify double mutants. The results matched the cuticle data: wg<sup>222</sup> double mutants showed the characteristic lawn of denticles (Figure 6F), while wg<sup>222</sup>; Df(3L)H99 double mutants (embryos without cells undergoing PCD as measured by TUNEL) had many more much smaller denticles (Figure 6E). In the course of this analysis, we also observed that Df(3L)H99 mutants have significantly more epidermal tissue in the head (as was previously observed by Grether et al. 1995) and in the lateral epidermis (Figure 6, J vs. K), consistent with the idea that wild-type embryos reduce the number of epidermal cells via PCD. We also examined whether reduction in PCD suppressed the weaker wg heteroallelic combination, wg<sup>222</sup>/Df(2)DE. We saw no noticeable suppression of the segment polarity phenotype and no noticeable increase in denticle number caused by either heterozygosity or homozygosity for Df(3L)H99 (data not shown). This may not be surprising as this weaker wg phenotype likely primarily reflects changes in cell fate without significant ectopic cell death, as the cuticle is nearly wild type in length.

**Blocking cell death in wg<sup>222</sup> increases cell number but decreases cell size:** The novel phenotype of wg<sup>222</sup>, Df(3L)H99 double mutants could have several causes. Extra denticles could result if individual cells secreted more denticles; alternately, they could result from an increased number of cells. To distinguish these possibilities, we examined the cell morphology of wild type, wg<sup>222</sup>, and wg<sup>222</sup>; Df(3L)H99 double-mutant embryos, using antibodies to phosphotyrosine to outline ventral epidermal cells and to label developing denticles. In wild-type embryos (Figure 6G), ventral epidermal cells form a reiterated pattern of denticle-secreting cells, which are very narrow in the anterior/posterior (A/P) axis, and naked cuticle-secreting cells, which are much less narrow. There are, on average, 12 rows of cells per segment. In contrast, in wg mutants there are only 8 rows of cells per segment (Figure 6H; the segment boundary was determined by comparison of the denticle pattern in cuticles to the phosphotyrosine pattern). In the wg<sup>222</sup>; Df(3L)H99 double mutant, cell number is greatly increased relative to the wg<sup>222</sup> single mutant. The double mutant has 12 to 14 rows of cells (Figure 6I), equaling or exceeding the number of cell rows in the wild type. Thus eliminating PCD in a wg<sup>222</sup> mutant embryo increases cell number, as expected.

Blocking cell death in a wg<sup>222</sup> mutant also had a second, unexpected consequence—cell size was significantly decreased. As mentioned above, in wild-type embryos anterior denticle-secreting cells are narrowed in the A/P axis, while posterior naked cuticle-secreting...
Figure 6.—Reducing PCD suppresses wg in a dose-sensitive fashion. (A–C) Cuticle preps. (D–F) Embryos labeled with phalloidin, which recognizes F-actin and thus highlights denticles. These embryos were also labeled via TUNEL (data not shown) to confirm their genotypes. (A, F) **wg** null mutants completely lack segment polarity and have only large, thick denticles. (B) **wg**<sup>bl22</sup>; **hid**<sup>070</sup>. (C, F) **wg**<sup>bl22</sup>; **Df(3L)H99**. (D) Wild type. In the double mutants, dentine number greatly increases. (G–K) Embryos stained with antiphosphotyrosine antibody to outline cells. (G) Wild type, showing reiterated groups of narrow cells, which will secrete denticles, and less narrow cells, which will secrete naked cuticle. There are 12 rows of cells per segment. (H) **wg** single mutants have fewer, larger cells. All cells are cuboidal, and there are about 8 rows of cells per segment. (I) **wg**<sup>1</sup>; **Df(3L)H99** double mutants have many more ventral epidermal cells than **wg** single mutants—there are 12 to 14 rows of cells per segment. Cells in the double mutant are much smaller. (J) Lateral view of a wild-type embryo during germ-band retraction. (K) **Df(3L)H99** at the same stage, revealing an increased number of cells compared to wild type. Excess cells form a lateral fold and ectopic folds near the maxillary and labial segments and toward the posterior. (L) Wild-type cuticle. (M) arm<sup>979</sup>. (N) arm<sup>979</sup>; UAS-dsh/VP16/armGAL4. Expressing high levels of **dsh** in arm<sup>979</sup> results in an increase in denticle number and reduction in denticle size, similar to that in **wg**<sup>bl22</sup>; **Df(3L)H99** double mutants.

In contrast, in a **wg** mutant all cells are both uniformly cuboidal (Figure 6H) and significantly larger than dentine-producing cells of a wild-type embryo. This increase in size likely reflects an increase in cell volume, because in optical cross sections **wg**<sup>bl22</sup> and wild-type cells were the same height (data not shown). In contrast, cells of **wg**<sup>bl22</sup>; **Df(3L)H99** double mutants are much smaller than those in **wg** single mutants (Figure 6I). Ventral cells of double mutants do resemble **wg**<sup>bl22</sup> single mutants in several ways; most cells are cuboidal, the cells create a pattern of block-like pseudosegments (though with more rows of cells than in **wg** single mutants), and all cells secrete denticles. We do not have a good explanation for the qualitative difference in the effect of **hid** on the arm and **wg**<sup>bl22</sup> phenotypes. We observed one other situation where manipulating Wg signaling resulted in an increased number of smaller denticles. Overexpression of **dsh** using the GALA-UAS system in an arm<sup>979</sup> mutant gives rise to a cuticle with many very small denticles, but with the length of an arm<sup>979</sup> single mutant (Figure 6, M vs. N). Dsh is a positive effector of Wg signaling mapping upstream of Arm in the Wg pathway; we imagine that Dsh overexpression slightly augments the residual Wg signaling in an arm zygotic mutant.

Our comparison of **wg**<sup>bl22</sup> and **wg**<sup>bl22</sup>; **hid** suggests that
an increase in PCD contributes to the reduced number of ventral epidermal cells in wg, consistent with previous observations (Pazdera et al. 1998). This might also explain the increased cell size in wg mutants, as epidermal cells have been observed engulfing dying neighbors (Pazdera et al. 1998), thus potentially increasing their size. However, since Wnts are mitogens in certain cell types (e.g., Dickinson et al. 1994; Neumann and Cohen 1996), we also considered an alternate explanation that could explain both reduction in cell number and increase in cell size in wg mutants: a failure to complete the normal program of cell division. Since no growth occurs within the embryo, as cells divide they are reduced in size, and thus if wg mutant cells failed to complete one round of mitosis, they would be twice as large.

We thus assessed the pattern of cell division in wg. Ventral epidermal cells divide three times after the blastoderm stage and arrest in G1 of the 17th embryonic cell cycle (Edgar and O'Farrell 1989). Cell divisions can be visualized by pulse labeling with BrdU to detect replicating nuclei or with an antibody that specifically recognizes a phosphoisoform of histone H3 that only occurs during mitosis (Figure 7; Su et al. 1998). Condensed mitotic chromosomes can be easily identified in fixed tissues with this antibody (Figure 7, C and D). We analyzed embryos at a time (stage 12) when cells of the ventral epidermis normally complete their 16th cell cycle, to see if wg mutants fail to undergo this last cell division. BrdU labeling indicated that ventral epidermal cells replicate during S phase 16 in wg mutants (data not shown). In addition, mitotic figures are as readily apparent in the ventral epidermis of stage 12 wg mutant embryos as they are in wild type (Figure 7, A and B). Thus, lack of Wg activity does not cause inappropriate cell cycle arrest. To compare the mitotic index between wild type and wg, we counted the total number of phosphohistone H3 positive nuclei of the ventral surface of individual embryos (arrow in Figure 7, C and D). There was no significant difference between the average number of mitotic cells in wild-type (175 ± 75.7; n = 7) vs. wg mutant (232 ± 44.2; n = 9) embryos. The variance in the absolute numbers of mitotic cells from embryo to embryo can be attributed to at least two factors: First, the precise age of each embryo scored differs slightly, as stage 12 spans ~2.5 hr of development at 25°C, and second, there is some cell cycle asynchrony among individual cells of a particular epidermal region that enter mitosis "together" (i.e., mitotic domains). We conclude that wg mutant embryos complete the normal number of cell divisions in the ventral epidermis, supporting the idea that the reduction in cell number in this region of late stage wg embryos is primarily a consequence of elevated levels of PCD.

**DISCUSSION**

Drosophila Arm and its human homolog βcat are multifunctional proteins that play roles in cell-cell adherens junctions and in the transduction of Wg/Wnt signals. In both roles, Arm/βcat acts as a scaffold upon which a multiprotein complex is assembled. In addition to these well-documented roles, Arm/βcat associates with other proteins, such as the EGF receptor (Hoschuetzky et al. 1994), fascin (Tao et al. 1996), and Presenilin 1 (Zhou et al. 1997). The biochemical function of these complexes is unknown.

We desire to learn more about the known roles of Arm in adherens junctions and in Wg signaling and also to begin to learn what Arm might do with its other partners. Genetics offers the opportunity to look for proteins that are functionally linked with Arm without assumptions as to their identity or biochemical role. Our initial goal in the screen was to identify novel proteins essential for adherens junction assembly or structure. However, as in all genetic screens, we had cast our net much wider. In the four cases where we proceeded from Deficiency to single gene, none encode new junctional proteins and each reveals a separate aspect of Arm biology. The fourth chromosome interactor dTCF revealed a previously unexpected role for a known component of the Wg pathway, providing evidence that dTCF not only activates Wg responsive genes but also, in the ab-
sence of Arm, represses them (Cavallo et al. 1998). Characterization of the interactor in 84E, Puckered, revealed a novel role for a known protein and led to data suggesting that the JNK and Arm pathways act in parallel both in dorsal closure and in ventral patterning (McEwen et al. 2000). The interaction with Dpresenilin suggests that the biochemical interaction observed in mammalian cells has impact on Arm function in vivo.

The fourth interactor, Hid, demonstrated that altering a downstream consequence of the loss of Wg signaling, programmed cell death, could suppress arm and, as is discussed below, suggested that Wg may act as a survival factor by modulating Hid activity.

We were initially concerned about the amount of labor required to screen for suppressors reducing the severity of an embryonic lethal phenotype without restoring viability (we expected that suppression to viability was unlikely). In retrospect, the screen, while labor intensive, was quite straightforward and could be applied to other embryonic lethal genes with a clear cuticle phenotype (arm's position on the X chromosome eased the effort). Use of the Deficiency kit reduced the number of stocks screened, although having completed the screen we now believe one could carry out such a screen using individual mutagenized lines. Others also recently screened for suppressors or enhancers of embryonic lethal phenotypes, suggesting that this approach may be widely applicable (Rapport et al. 1995; Hudson et al. 1998; A. Bejsovec, personal communication).

Our screen had several limitations that affected the spectrum of genes identified. First, for a gene to be identified, it had to affect the arm phenotype in a dose-sensitive way. Second, the effect on arm had to be consistent and substantial. Our arbitrary cutoff for degree of interaction likely eliminated genes in the desired categories in which mutations did not sufficiently suppress arm. For example, loss-of-function mutations of Drosophila ablision or Deficiencies that remove it suppress arm, but not to a sufficient degree to be scored positive in our screen (Loureiro and Peifer 1998). Third, due to the allele of arm we chose, we could not reliably score enhancement of the segment polarity phenotype, and likewise, potentially due to high levels of maternal Arm, we did not detect any interactor that produced defects in epithelial integrity. Fourth, since many P-element alleles are not null, our ability to move from Deficiency to single gene using these mutations was consequently limited. Finally, genetic background may obscure some interactions—for example, we saw a clear genetic interaction with two alleles of Dpresenilin but saw no significant interaction with two Deficiencies that remove it.

During preparation of this manuscript, an article appeared describing a different strategy for identifying genetic interactors with arm, which provides an interesting comparison. Greaves et al. (1999) expressed in the posterior compartment of the wing the intracellular domain of DE-cadherin, which they previously found could sequester Arm and thus block its signaling activity (Sanson et al. 1996). In parallel they overexpressed Arm in the same place. Each caused a reproducible wing phenotype, which appears to reflect reduction and elevation of Wg signaling, respectively. They then screened for modifiers of these phenotypes using, as we did, the Deficiency kit. Many of the deficiencies tested thus overlapped (though not all, as we did not test the X chromosome and they did not test the fourth chromosome).

We compared the spectrum of modifying Deficiencies obtained in our screen with the 59 interacting Deficiencies identified in their screen. The Deficiencies identified were quite different, likely reflecting the distinct methods used to examine interaction and the different tissues involved. These differences illustrate the benefit of taking a variety of genetic approaches to modifier screens and emphasize that no one screen will identify all or even most potential interactors. Most interacting Deficiencies identified in their screen did not interact in our screen; for 39 of their interacting deficiencies, the percentage of suppression in our screen was <3%. Eight of their interacting Deficiencies were weak interactors in our screen [Df(2L)scl19-8, Df(2L)prd1.7, Df(2L)j32, Df(2L)H120, Df(3L)Jn5, Df(3L)zn47, Df(3R)eb87-5, and Df(3R)Hu]. Four interacting Deficiencies from their screen, Df(3L)Spd, Df(3L)Cat, Df(3R)D1-BX12, and Df(3R)p712, were strong interactors in our screen. Within two of these latter regions we identified interactors: hid from Df(3L)Cat and puc from Df(3R)p712.

Even in cases where the two screens identified the same Deficiency, it is not clear that the same gene is responsible. First, in several cases different subsets of overlapping Deficiencies interacted in the two screens. Second, Greaves et al. (1999) identified interacting genes in many of their Deficiencies. In four cases, we also examined those candidates. Two were identified as interactors in our screen as well (DE-cadherin and zw3, used in our reconstruction experiments). In contrast, one of their interactors, ug, did not interact in our screen, even though a Deficiency that removes it, Df(3L)Spd, did interact. Likewise, components of the EGFR pathway interacted in their tests but not ours. Finally, in our hands naked complements Df(3L)Cat, thus ruling it out as our interactor in that region; in this region we identified hid as the interactor.

Hid activity, PCD, and the segment polarity phenotype: It has been known for more than a decade that PCD plays an important role in the segment polarity phenotype resulting from inactivation of either the Hedgehog or Wg pathways (Martinez-Arias 1985; Klingensmith et al. 1989). Recently, Minden and colleagues carried out a detailed analysis of this process, quantitating cell death in ug, arm, gooseberry, and naked. They found that the elevation in cell death affected particular cells (Pazdera et al. 1998). Since the first reports of cell death in segment polarity mutants, the
machinery that drives PCD in embryos has begun to be identified. Homozygosity for the small chromosomal
Deficiency, \(D'(3L)H99\), blocks essentially all PCD
(White et al. 1994). Within this interval, three genes
play roles in PCD: grim, reaper, and hid (reviewed in
Abrams 1999). Ectopic expression of any of these can
trigger PCD, but loss-of-function mutations are only
available for hid.

Given the role of PCD in the segment polarity pheno-
type, it is perhaps not surprising that elimination of
PCD would alter it. Several aspects of the effect of PCD
reduction were unexpected, however. First, and most
striking, the phenotypes of arm and \(wg\) mutants were
very sensitive to relatively small changes in the dose of
hid and the other cell-death promoters. For example,
while heterozygosity for hid has no known effects on
normal development, it strongly suppresses arm. Further
reductions in the levels of hid or the other cell-death
regulators had no additional effect on arm, suggesting
that reducing the Hid dose by half eliminated the rele-
vant ectopic PCD that occurs in an arm mutant. The \(wg\)
phenotype was also suppressed in a highly dose-sensitive
fashion, but in a different dosage range. A 50\% reduc-
tion of hid caused slight but detectable effects, a 50\%
reduction in all three death promoters caused greater
suppression, while homozygosity for the deletion remov-
ing all three genes resulted in the strongest \(wg\) suppres-
sion.

Recent observations regarding the role of Hid in PCD
in the eye may explain this. Signaling through the ras/
mitogen-activated protein kinase (MAPK) pathway pro-
motes cell survival by antagonizing Hid (Bergmann et
al. 1998; Kurada and White 1998). These authors sug-
gested that Hid serves as a rheostat, with its levels de-
dtermining the probability of PCD. They further suggest
that Hid activity has to exceed a threshold to trigger
PCD; the accumulation of hid mRNA in cells that are
not programmed to die is consistent with this (Grether
et al. 1995). Our observations further support this
model. Wg signaling may normally antagonize Hid, poten-
tially by regulating its expression. In embryos where
Wg signaling is attenuated, elevated Hid activity may
trigger PCD when it rises above a critical threshold. A
threshold model could explain why the segment polarity
phenotype is so sensitive to the dose of Hid and its
partners.

Another surprise was the qualitative difference in the
effect of cell death reduction on \(wg\) and arm mutants.
While the resulting cell number was likely increased in
both double-mutant genotypes in the arm; hid double
mutant, the reduction in PCD restored an almost wild-
type-length cuticle, while in the \(wg; hid\) double mutant,
the increase in cell number was not reflected in an
increase in cuticle length. The reason for this remains
mysterious. One possible explanation for this discrep-
ancy is the difference in the degree to which Wg signal
is compromised in the two situations and the embryonic
stage at which this disruption occurs. In the \(wg\) null,
Wg signaling is totally eliminated from the begin-
ing of development. In contrast, perdurance of maternal
Arm substantially rescues early defects in Wg signaling
in \(arm\) zygotic nulls (Klingensmith et al. 1989). \(arm\)
mutants remain more normal in morphology than \(wg\)
mutants through the onset of germband retraction and
retain remnant denticle diversity. Thus when one elimi-
nates PCD in an \(arm\) mutant a more normal pattern is
restored. The difference in amount and timing of Wg
signaling in the two backgrounds may also explain why
\(arm\) mutants are affected by smaller alterations in Hid
level. The remaining Wg signaling in an \(arm\) zygotic
mutant may promote cell survival to some extent, mean-
ning that a smaller reduction in Hid activity prevents
ectopic PCD.

We were also surprised that reduction in cell death
alleviated \(arm\)'s dorsal closure defect. We previously sus-
ppected that this defect was due solely to Arm's role
as a catenin. However, recent data suggest that dorsal
closure is promoted by Wg signaling (McEwen et al.
2000). We now suspect that defects in Wg signaling and
catenin function combine to block dorsal closure in \(arm\)
mutants. Restoring either rescues the \(arm\) dorsal closure
defect. However, blocking PCD alone should not restore
Wg signaling or catenin function. Perhaps the excess
cell death in the head region or in the amnioserosa of
an \(arm\) mutant contribute to its dorsal closure defect.

Presenilins and Arm function: While evaluating the
effectiveness of our screen, we tested a variety of candi-
date genes, including some that mapped within noninter-
acting Deficiencies. Heterozygosity for one of these,
D\(presenilin\), strongly suppressed \(arm\). Presenilins form a
family of multipass transmembrane proteins that were
first identified because missense mutations in two hu-
mans Presenilins cause early onset familial Alzheimer
disease (FAD; reviewed in Haass and Strooper 1999;
Nishimura et al. 1999b). The cell biological func-
tion of Presenilins and how dysfunction contributes to
disease remain controversial. Genetic data in Caenorhab-
ditis elegans and Drosophila implicate Presenilins in the
function of Notch proteins, most likely via effects on
protein processing. Likewise, human Presenilin muta-
tions affect proteolytic processing of the plaque protein
\(A\beta\); this may lead to pathology (reviewed in Haass
and Strooper 1999). Recently, it was found that both
\(\beta\)cat and other Arm repeat proteins such as \(\delta\)-catenin
associate with Presenilins in vivo. The function of this
interaction remains confusing. Zhang et al. (1998)
reported that wild-type Presenilin stabilizes \(\beta\)cat and that
this is abrogated by missense mutations found in FAD
patients, and Nishimura et al. (1999a) reported that
\(presenilin\) missense mutant cells from FAD patients have
less nuclear \(\beta\)cat. These data support a role for Preseni-
linss as positive regulators of Wnt signaling via Arm/
\(\beta\)cat. In contrast, both Muryama et al. (1998) and Kang
et al. (1999) report that overexpression of wild-type Pre-
serlin destabilizes βcat: Kang et al. further show that βcat is stabilized in both Presenilin1 null fibroblasts or if FAD mutants of Presenilin1 are overexpressed, while Murayama et al. demonstrate that a Wnt-responsive promoter is downregulated by Presenilin overexpression. These data support a conclusion opposite from that above, in which wild-type Presenilins negatively regulate Wnt signaling. Finally, Georgakopoulos et al. (1999) suggest that the presenilin-βcat complex includes cadherins, in contravention of most other data. Our genetic data are most consistent with a model in which Presenilins negatively regulate Wg signaling (Figure 3) either directly or indirectly by binding Arm/βcat or by influencing adherens junction assembly. Clearly much work remains to differentiate between the different possible mechanisms.

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LITERATURE CITED


Nüsslein-Volhard, C., and E. Wieschaus, 1980 Mutations affect-
Suppressors of Drosophila armadillo

1. Suppressors of Drosophila armadillo


13. The Drosophila caspase inhibitor DIAPI is essential for cell survival and is negatively regulated by HID. Cell 98: 455-463.


Communicating editor: K. Anderson
## APPENDIX A

### Refining strongly interacting regions

<table>
<thead>
<tr>
<th>Interacting deficiencies</th>
<th>Original region</th>
<th>Defining regions</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(2L)sc19-5; Dp(2;1)B19</td>
<td>25A4-5;25D5-7</td>
<td>24C2-8;25C8-9</td>
<td>+Weak</td>
</tr>
<tr>
<td>Df(2L)sc19-8; Dp(2;1)B19</td>
<td></td>
<td>25D2-4;26B2-5</td>
<td>+Weak</td>
</tr>
<tr>
<td>Df(2L)ejl5</td>
<td></td>
<td>25D2-4;25D6E1</td>
<td>—</td>
</tr>
<tr>
<td>Df(2L)tv2</td>
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</tr>
<tr>
<td><strong>Df(2L)spd</strong></td>
<td>27D-E;28C</td>
<td>27C2-9;28B3-4</td>
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<tr>
<td>Df(2L)J136-H152</td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td><strong>Df(2L) TE29Aa-11</strong></td>
<td>28E4-7;29B2-C1</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Df(2L)TW137; Dp(2;2) M(2) m[+]</td>
<td>36C2-4;37B9-C1</td>
<td>36A8-9;36E1-E2</td>
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<tr>
<td>Df(2L)H20</td>
<td></td>
<td>36D1-E1;36F1-37A1</td>
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<td>Df(2L)M36F5; Dp(2;2) M(2) m[+]</td>
<td></td>
<td>36E4-F1;38A67</td>
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<tr>
<td>Df(2L)TW50</td>
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<td></td>
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<tr>
<td><strong>Df(2R)ST1</strong></td>
<td>42B3-5;43E15-18</td>
<td>42E,44C</td>
<td>—</td>
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<tr>
<td>Df(2R)cm9</td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td><strong>Df(2R)PC4</strong></td>
<td>55C1-3,55E2-4</td>
<td>55E2-4;56C1-11</td>
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<tr>
<td>Df(2R)P24</td>
<td></td>
<td>54F6-55A1;55C1-3</td>
<td>—</td>
</tr>
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<td>Df(2R)J111B</td>
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<tr>
<td><strong>Df(2R)017</strong></td>
<td>45B6;56F15;+ In56D-E;58E-F</td>
<td>56F9-17;57D11-12</td>
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<tr>
<td>Df(2R)AA21</td>
<td></td>
<td></td>
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<tr>
<td><strong>Df(3L)W10</strong></td>
<td>75A6-7;75C1-2</td>
<td>75B8;75F1</td>
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<tr>
<td><strong>Df(3L)Cat</strong></td>
<td>75B8;75F1</td>
<td>75A6-7;75C1-2</td>
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<td><strong>Df(3L)FeMK</strong></td>
<td>78A2;78C1-5</td>
<td>78C1-5;78C9-D3</td>
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<td><strong>Df(3L)Pc</strong></td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td><strong>Df(3R)Scr</strong></td>
<td>84A1-2;84B1-2</td>
<td>83E1-2;84A4-5</td>
<td>+Weak</td>
</tr>
<tr>
<td><strong>Df(3R)WIN11</strong></td>
<td>83E1-2;84A4-5</td>
<td></td>
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</tr>
<tr>
<td><strong>Df(3R)GA3</strong></td>
<td>84F2-85A5-7</td>
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<tr>
<td><strong>Df(3R)Tpi10, Tp(3;3)Dfd[rv1]</strong></td>
<td>83C1-2;84B1-2</td>
<td>84A1-2;84A4-5</td>
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</tr>
<tr>
<td><strong>Df(3R)MAP2</strong></td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td><strong>Df(3R)p712</strong></td>
<td>84D4-6;85B6</td>
<td>843-5;84F1-2</td>
<td>Strong</td>
</tr>
<tr>
<td><strong>Df(3R)d7</strong></td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td><strong>Df(3R)d1-bx12</strong></td>
<td>91F1-2;92D3-6</td>
<td>91A;91F5</td>
<td>—</td>
</tr>
<tr>
<td><strong>Df(3R)Che7</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>C(4)RM</strong></td>
<td>101-104</td>
<td>101F1-102B</td>
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<tr>
<td><strong>Df(4)M62f</strong></td>
<td></td>
<td></td>
<td>—</td>
</tr>
</tbody>
</table>

Boldface, original deficiency.

* These two deficiencies fail to complement.

+ Narrowed each other.

* Interaction most likely more distal than original breakpoints.

## APPENDIX B

### Weakly interacting regions

<table>
<thead>
<tr>
<th>Weak Interactors</th>
<th>Breakpoints</th>
<th>% supp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(2L)a1</td>
<td>21B8-C1;21C8-D1</td>
<td>4.8</td>
</tr>
<tr>
<td>Df(2L)S32</td>
<td>23C5-5;23D1-2</td>
<td>3.8</td>
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<tr>
<td>Df(2L)sc19-8; Dp(2;1)B19</td>
<td>24C2-8;25C8-9</td>
<td>3.0</td>
</tr>
<tr>
<td>Df(2L)N22-3</td>
<td>30A1-2,30D1-2</td>
<td>5.7</td>
</tr>
<tr>
<td>Df(2L)prdl2.7</td>
<td>33B2-3;34A1-2</td>
<td>3.8</td>
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<td>Df(2L)rl0</td>
<td>35E1-2;36A6-7</td>
<td>5.4</td>
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<td>Df(2L)H20</td>
<td>36A8-9;36E1-2</td>
<td>4.1</td>
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<td>Df(2L)TW84</td>
<td>37F5-38A1;39D3-E1</td>
<td>3.8</td>
</tr>
<tr>
<td>Df(2R)M41A4</td>
<td>41A</td>
<td>3.6</td>
</tr>
<tr>
<td>In(2R)hwc[VDe21] Cy[R]/In(2LR)Gla</td>
<td>41AB;42A2-3</td>
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<tr>
<td>Df(2R)vg135</td>
<td>49A-B;49D-E</td>
<td>3.7</td>
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<td>Df(2R)Px2</td>
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<td>Df(2R)Es1</td>
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<td>Df(3L)ZN47</td>
<td>64C;65C</td>
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<td>Df(3L)fz-GF3b</td>
<td>70C1-2;70D4-5</td>
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<td>Df(3R)Hu</td>
<td>86A6-B1;86B3-6</td>
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</tr>
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<td><strong>Df(3R)crb87-5</strong></td>
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<td><strong>Df(3R)B83</strong></td>
<td>95F7;96A17-18</td>
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<td></td>
<td>99C8;100F5</td>
<td>3.5</td>
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