Erratum

The e-press version of this article that was published on 23rd August 2005 contains two errors on p. 4128.

In the legend to Fig. 6, the penultimate sentence should read: Aggregation was assessed by microscopy.

In the right-hand column, paragraph 2, the last sentence should read: It is not known whether similar changes to the βA strand in cadherins would modulate intramolecular docking of Trp2 and alter the free energy balance in favour of strand exchange.

Both the published print and online versions of this article are correct.

We apologise to the authors and readers for these mistakes.

Cadherin adhesion depends on a salt bridge at the N-terminus

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Summary

There is now considerable evidence that cell adhesion by cadherins requires a strand exchange process in which the second amino acid at the N-terminus of the cadherin molecule, Trp2, docks into a hydrophobic pocket in the domain fold of the opposing cadherin. Here we show that strand exchange depends on a salt bridge formed between the N-terminal amino group of one cadherin molecule and the acidic side chain of Glu89 of the other. Prevention of this bond in N-cadherin by introducing the mutation Glu89Ala or by extending the N-terminus with additional amino acids strongly inhibited strand exchange. But when the two modifications were present in opposing cadherin molecules respectively, they acted in a complementary manner, lowering activation energy for strand exchange and greatly increasing the strength of the adhesive interaction. N-cadherin that retained an uncleaved prodomain or lacked Trp2 adhered strongly to the Glu89Ala mutant but not to wild-type molecules. Similarly, N-cadherin in which the hydrophobic acceptor pocket was blocked by an isoleucine side chain adhered to a partner that had an extended N-terminus. We explain these results in terms of the free energy changes that accompany strand exchange. Our findings provide new insight into the mechanism of adhesion and demonstrate the feasibility of greatly increasing cadherin affinity.

Key words: Cadherin, Salt bridge, Strand exchange, Tryptophan, N-terminus

Introduction

Cadherins are a large family of cell surface calcium-dependent adhesion molecules that include the classical (Type I) cadherins, non-classical (Type II) cadherins, the desmogleins and desmocollins, and the protocadherins α, β and γ (Frank and Kemler, 2002; Nollet et al., 2000). Cadherins are essential for the structural integrity of all vertebrate solid tissues and they determine cell-cell recognition during morphogenesis and have signalling functions that influence cell migration and differentiation (Cavallaro and Christofori, 2004; Hirano et al., 2003; Thiery, 2003; Wheelock and Johnson, 2003). Cadherin molecules consist of a series of extracellular β-barrel domains, a transmembrane domain and a long cytoplasmic domain. The extracellular region is stiffened into a curved rod-like structure by calcium coordinated in the domain junctions (Boggon et al., 2002). Although the mechanism of cadherin adhesion has long been controversial, it is widely agreed that the most distal domain, domain 1, is essential for adhesion (Klingelhofer et al., 2000; Shan et al., 2004) and that the second amino acid at the N-terminus, Trp2, is particularly important (Kitagawa et al., 2000; Tamura et al., 1998). Crystal structures and NMR studies of cadherins have identified several putative contact surfaces that could represent an adhesive interface (Boggon et al., 2002; Haussinger et al., 2002; Pertz et al., 1999; Shapiro et al., 1995; Tamura et al., 1998). The most convincing shows a structure in which Trp2 cross-intercalates into a hydrophobic pocket in the opposing domain, a mutual process referred to as βA strand exchange (Boggon et al., 2002; Haussinger et al., 2004; Shapiro et al., 1995). Recent work in our laboratory (Harrison

et al., 2005) has provided persuasive evidence that strand exchange is a primary event in cadherin-mediated cell adhesion. The exchange process is an example of the '3D domain swap' mechanism for oligomerization that has been described for a heterogeneous group of proteins and involves mutual exchange of one or more structural components (Liu and Eisenberg, 2002; Rousseau et al., 2003; Yang et al., 2004).

It has long been known that correct post-translational processing of cadherin molecules is essential for adhesion (Ozawa and Kemler, 1990). Type I cadherins are synthesised with a prodomain of more than 100 amino acids, which has the structure of a typical cadherin fold (Koch et al., 2004). There is an unstructured linker of approximately 30 amino acids between the prodomain and the first domain, EC1, of the mature molecule. A multi-basic recognition motif is cleaved by furin proteases to give the mature cadherin molecule that has a conserved tryptophan as the second amino acid from the N-terminus. Failure to remove the prodomain prevents adhesion (Koch et al., 2004; Ozawa and Kemler, 1990). The presence of even a few additional amino acids at the N-terminus completely ablates adhesive function (Corps et al., 2001; Ozawa and Kemler, 1990). In keeping with this observation, a recent NMR study (Haussinger et al., 2004) showed that correct processing at the N-terminus was required for the strand exchange mechanism or for intramolecular docking of Trp2 into its own domain. The crystal structure of C-cadherin, in which the N-terminus is correctly processed, shows that strand exchange brings the amino group of Asp1 in close proximity to the acidic side chain of a conserved amino acid, Glu89, in the opposing

cadherin domain, suggesting that a salt bridge could form here to stabilise Trp2 docking (Boggon et al., 2002). The significance of the putative salt bridge has been questionable because crystal structures of E- and N-cadherins show Trp2 integrated into the domain fold despite extension of the N-terminus and, consequently, the absence of this ionic bond (Pertz et al., 1999; Schubert et al., 2002; Shapiro et al., 1995).

In the present report we have investigated the significance of the salt bridge in cell adhesion mediated by N-cadherin. We have prevented formation of the bond in one or both components of the adhesive dimer by extending the N-terminus or by mutating Glu89 to Ala. The results demonstrate with striking clarity that the salt bridge plays a vital role in adhesion by stabilising Trp2 docking and that intramolecular and intermolecular docking of Trp2 are in dynamic equilibrium. When the Glu89Ala mutation and the N-terminal extension are present in opposing cadherin molecules respectively, they form a complementary pair, each preventing intramolecular docking of Trp2 but facilitating strand exchange in one direction. In these circumstances the normal equilibrium is disturbed and the strength of cadherin adhesion is greatly increased.

Materials and Methods

Preparation of DNA constructs and their transfection into cell lines

Mutations were prepared in full-length chicken N-cadherin cDNA in pcDNA3.1 using the QuikChange mutagenesis method (Stratagene). Constructs were stably transfected into K562 lymphomyeloid cells as previously described (Harrison et al., 2005) as these cells have been shown to lack natural expression of cadherins. Clonal cell lines were obtained by limiting dilution and selected for equal expression of N-cadherin. Mutant and wild-type chicken N-cadherin Fc fusion proteins containing the five extracellular domains were prepared, standardised and quantified as previously described (Harrison et al., 2005). All cell lines were cultured in DMEM with 10% FCS containing G418 at 1 mg/ml.

Cadherin-mediated cell adhesion

Adhesion tests were conducted as described in previous reports (Corps et al., 2001; Harrison et al., 2005). Briefly, K562 cells or L cells transfected with wild-type or mutant N-cadherin were allowed to settle for 45 minutes at 37°C onto N-cadherin Fc fusion proteins coated at 1 μg/ml to a 96-well plate. Non-adherent cells were then washed off and residual adherent cells were quantified by measuring acid phosphatase activity. Assays were conducted in quadruplicate and results are expressed as the percentage of cells adhering ±s.e.m.

Bead aggregation assay

Dynabeads (Dynal Biotech) coupled to Protein A were coated with N-cadherin Fc at 1 μg/ml in calcium- and magnesium-free HBSS containing 0.1% Tween 20, 1% FCS and 4 mM EDTA. Eppendorf tubes containing beads and fusion protein were rotated slowly for 1 hour at room temperature to allow binding to take place. The beads were then washed in the above assay buffer lacking EDTA and then resuspended in the same buffer supplemented with 1.25 mM $CaCl_2$. Beads were allowed to aggregate in a volume of 100 μl for 2 hours at 37°C by slow rotation, in an Eppendorf tube, at approximately 20 rpm. Aggregation was then assessed by light microscopy.

Immunofluorescent staining of K562 transfectants

Cells were stained for chicken N-cadherin using antibody NCD-2 (R&D Systems) at 5 μg/ml. The secondary antibody was FITC-labelled goat anti-rat IgG (Serotec, UK). For staining transfectants with N-cadherin Fc fusion proteins, the cells were treated with the fusion proteins at 5 μg/ml for 90 minutes on ice in Hanks balanced salt solution (HBSS) containing 2% FCS and 0.1% sodium azide. After washing, bound fusion protein was detected with FITC-labelled goat anti-human Fc (Serotec) and quantified by flow cytometry using a FACSCalibur (Becton Dickinson)

Cleavage of the N-cadherin prodomain

L cells expressing mouse N-cadherin with an uncleaved prodomain were a kind gift of Weisong Shan (Montreal Neurological Institute, Canada). The normal furin cleavage site, RQKR, had been replaced with a Factor Xa site, IEGR, to give the correct N-terminus after digestion. Trypsin also cleaved at this position (Koch et al., 2004) and proved to be more efficient than Factor Xa. L cells suspended in HBSS containing 0.1% BSA were treated with 0.01% trypsin (Sigma, Type XI) in the presence of 2 mM Ca^{2+} for 10 minutes at 37°C and the digestion was then quenched with soya bean trypsin inhibitor, 0.5 mg/ml (Sigma, Type I-S). Cells were then washed and tested for adhesion to N-cadherin Fc fusion protein. To check for complete removal of the prodomain, the cells were lysed in SDS sample buffer and the cadherin analysed by SDS-PAGE under reducing conditions on a 4-12% gradient gel. N-cadherin was identified by western blotting using rabbit anti-pan cadherin antiserum specific for the cytoplasmic domain (Sigma, code C3678) followed by affinity-purified HRP-labelled sheep anti-rabbit IgG, F(ab′)$_2$-specific (Serotec).

Viewing molecular structures

Cadherin structures were displayed using Swiss PDB Viewer (http://www.expasy.org/spdbv/).

Results

Disrupting the salt bridge between the N-terminus and Glu89

Two salt bridges are formed during mutual strand exchange in cadherin (Fig. 1a). To prevent formation of these bonds, Glu89 of N-cadherin was mutated to Ala or, alternatively, the N-terminus was extended by adding two glycine residues to Asp1 to displace the N-terminal amino group away from the acidic side chain of Glu89. Mutant N-cadherin proteins were expressed in K562 lymphomyeloid cells that were matched for equal cell surface expression of the respective cadherins (Fig. 1b). Transfectants were tested for adhesion to mutant or wild-type N-cadherin Fc fusion proteins (Fig. 2). Disruption of the salt bridge by either the Glu89Ala mutation or the double glycine N-terminal extension strongly inhibited adhesion to wild-type N-cadherin. Similarly, each of the two mutants failed to adhere to their own kind. In sharp contrast, the combination of the Glu89Ala mutation on one side of the adhesive pair with the Gly-Gly extension on the other resulted in markedly stronger adhesion than that given by wild-type N-cadherin, a high proportion of the cells becoming flattened to the assay plate. The double mutation Glu89Ala plus the Gly-Gly extension in the same molecule prevented adhesion in all circumstances. A negative control to demonstrate that the assay reflected cadherin-mediated adhesion was provided by the N-cadherin Fc mutant Asp134Ala. This mutation prevents coordination of the third calcium atom, Ca3, in the junction between domains 1 and 2 and is known to prevent cadherin-
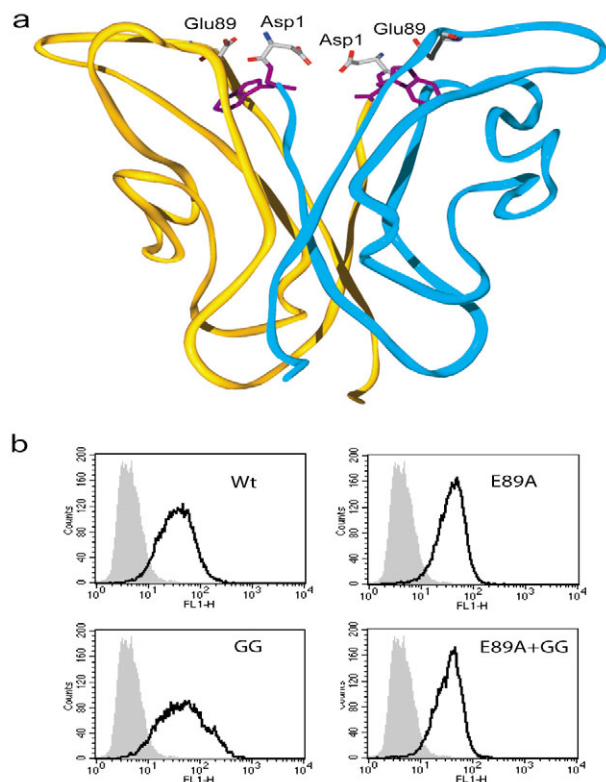
Fig. 1. The salt bridge between Asp1 and Glu89. (a) Strand exchange in the C-cadherin structure 1L3W. The two domain 1 protomers are shown in blue and yellow respectively and Trp2 is coloured purple. Juxtaposition of the nitrogen of the N-terminal amino group of Asp1 (blue) and the oxygen atoms of the acidic side chain of Glu89 (red) are shown. (b) Cell surface staining of mutant and wild-type (Wt) N-cadherins expressed by K562 transfectants, matched for equal expression. The mutations Glu89Ala (E89A) and Gly-Gly (GG; in which the N-terminus was extended by two glycine residues) were present singly or together in the same molecule. The shaded profile is a negative control using FITC-labelled second antibody only.

mediated adhesion (Corps et al., 2001; Ozawa et al., 1990). The Asp134Ala mutation alone (Fig. 2a) or in combination with Glu89Ala or with the Gly-Gly N-terminal extension (Fig. 2b) reduced adhesion to background levels. The enhanced adhesion seen with the complementary pair of mutants Glu89Ala and the Gly-Gly extension was confirmed using a range of concentrations of N-cadherin Fc coated to the assay plate (Fig. 2c).

Affinity between the Glu89Ala and Gly-Gly extension mutants

To investigate whether the enhanced adhesion observed with this complementary pair reflects an increase in affinity, we tested whether soluble N-cadherin Fc fusion proteins would bind to cell surface N-cadherin using a protocol similar to immunofluorescent staining by antibodies. Binding between wild-type N-cadherin molecules was undetectable (Fig. 3). In contrast, the interaction between the Glu89Ala mutant and the Gly-Gly extension mutant gave strong cell surface staining, approaching that seen with antibodies to cadherins. Previous
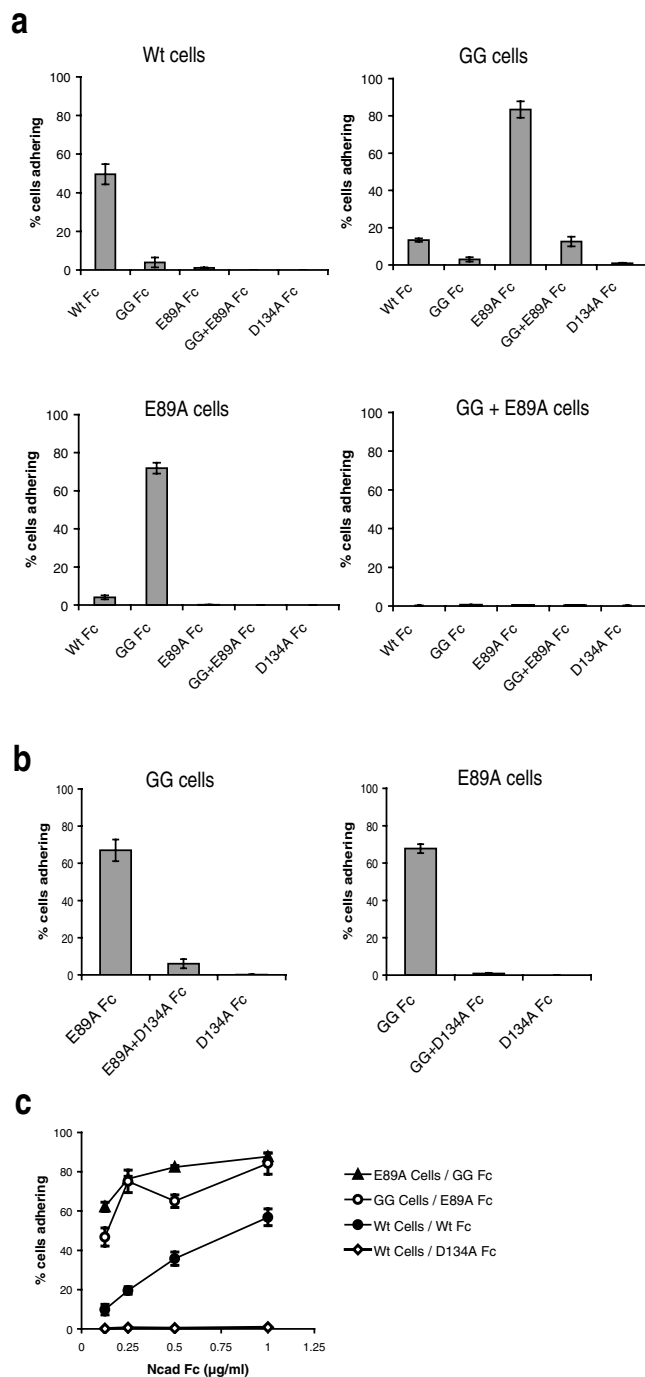

Fig. 2. Effect of the salt bridge on cadherin-mediated cell adhesion. (a) Adhesion of N-cadherin transfectants to wild-type or mutant N-cadherin Fc fusion proteins coated at 1 µg/ml. Extension of the N-terminus by two amino acids (GG) or the mutation Glu89Ala (E89A) inhibited adhesion to wild-type N-cadherin but, when present in opposing molecules, they formed a complementary pair resulting in enhanced adhesion. The mutation Asp134Ala (D134A) prevents coordination of calcium in the junction between domains 1 and 2 and served as a negative control. (b) Adhesion between Glu89Ala and the Gly-Gly extension mutant was ablated by the mutation Asp134Ala. (c) Enhanced adhesion between the complementary pair, Gly-Gly and Glu89Ala, compared with adhesion between wild-type N-cadherin molecules over a range of concentrations of the Fc fusion proteins.
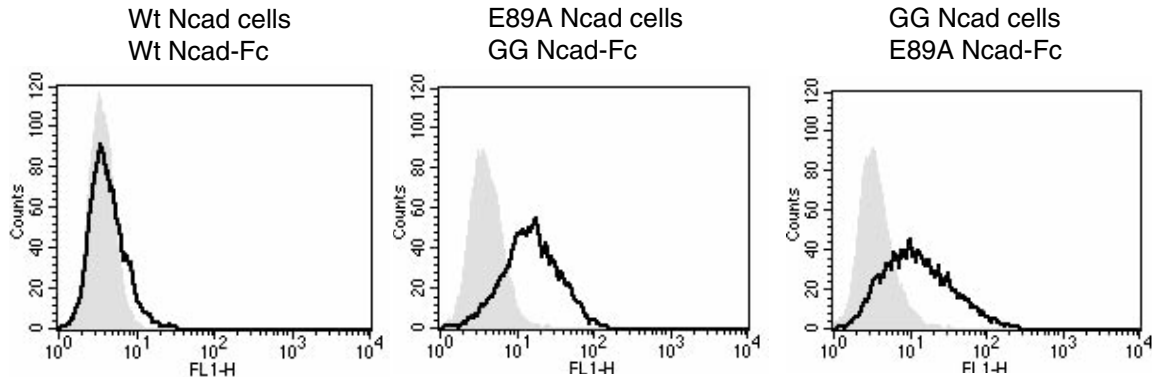
Fig. 3. Binding of soluble N-cadherin Fc to cell surface N-cadherin. Mutant or wild-type Fc fusion proteins were used to 'stain' cell surface N-cadherin expressed by K562 transfectants. Binding was detected with FITC-labelled anti-Fc. The shaded profiles are negative controls using N-cadherin Fc with the mutation Asp134Ala. Results show that the affinity between wild-type cadherin molecules was too low to give detectable binding, whereas the interaction between Glu89Ala (E89A) and the Gly-Gly mutant (GG) gave strong staining.

studies (Baumgartner et al., 2000; Haussinger et al., 2004) measuring affinity between normal trans-acting cadherin molecules have reported K_D values in the range 10^{-3} to 10^{-5} M. Although we have not yet obtained precise measurements of affinity between the complementary cadherin mutants, the present data clearly show a large increase in affinity compared with that between wild-type molecules.
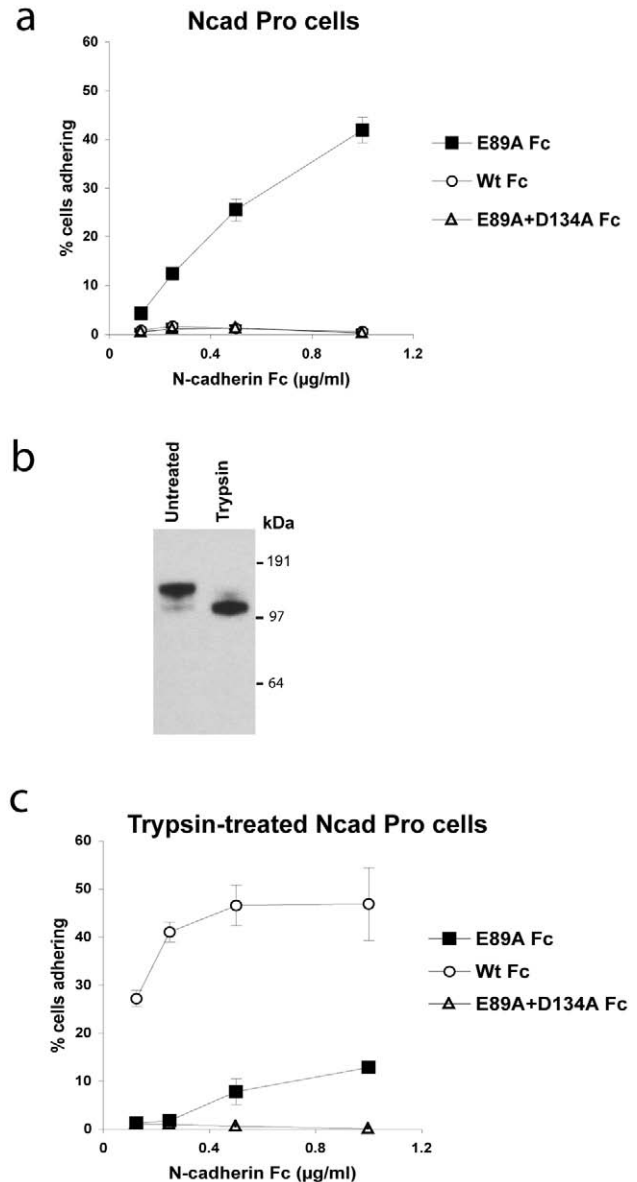
Effect of an uncleaved prodomain

To investigate whether an uncleaved prodomain has the same effect as a two amino acid extension to the N-terminus, we tested the ability of L cell transfectants expressing unprocessed N-cadherin to adhere to the Glu89Ala mutant. There was strong adhesion of the transfectants to the Glu89Ala Fc fusion protein but no adhesion to wild-type N-cadherin or to the Asp134Ala negative control (Fig. 4a). A Factor Xa cleavage site introduced into the transfected N-cadherin allowed removal of the prodomain from the cell surface with either Factor Xa or trypsin. Trypsin proved to be more efficient, almost completely removing the prodomain (Fig. 4b). In these circumstances the cells acquired the ability to adhere to wild-type N-cadherin whereas adhesion to the Glu89Ala mutant was greatly reduced (Fig. 4c).

Model for strand exchange

Our proposed model for strand exchange is illustrated in Fig. 5. First, the N-cadherin domain 1 is considered in isolation (Fig. 5a). In keeping with a previous report from our laboratory (Harrison et al., 2005) and a recent NMR study of E-cadherin (Haussinger et al., 2004), there is a dynamic equilibrium between docked and un-docked Trp2 that favours the docked

Fig. 4. Effect of the prodomain. L cells expressing N-cadherin with an uncleaved prodomain were tested for adhesion to mutant or wild-type N-cadherin Fc fusion proteins. (a) L cells adhered to the Glu89Ala (E89A) mutant but not to wild-type N-cadherin. (b) Western blot demonstrating removal of the prodomain by treatment of the L cell transfectants with trypsin. (c) After treatment, the L cells adhered strongly to wild-type N-cadherin whereas adhesion to the Glu89Ala mutant was greatly diminished.
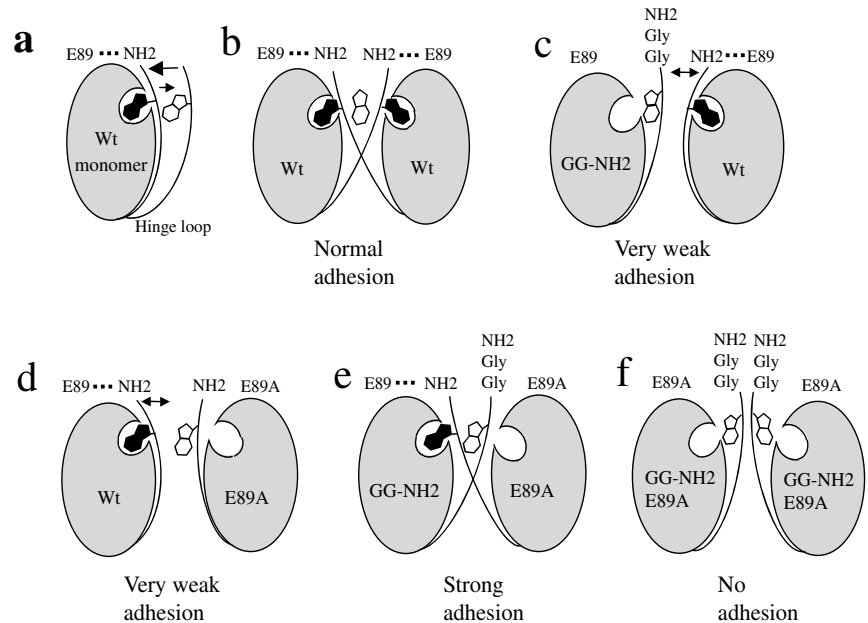
Fig. 5. Proposed role of the salt bridge in the strand-swap mechanism in mutant and wild-type N-cadherin. A full explanation is offered in the text. (a) Domain 1 in isolation. An equilibrium between docked and undocked Trp2 favours the docked form because the salt bridge between Glu89 and the N-terminus (shown as a dotted line) stabilises Trp2 insertion. (b) Adhesion between wild-type molecules. A transition state in which Trp2 is undocked is sampled from either side and is depicted as an 'unshaded' tryptophan. Adhesion is moderate. (c) The salt bridge on one side is prevented by extension of the N-terminus. (d) The salt bridge is prevented by the mutation Glu89Ala. In both c and d, adhesion is very weak. Although one strand can cross-intercalate, the process competes unfavourably with intramolecular docking of Trp2 into the wild-type domain. (e) The two mutations form a complementary pair. Intramolecular docking is prevented and therefore the activation barrier for strand exchange is lowered. Exchange of one strand is possible and cross-intercalation of Trp2 is stabilised by the salt bridge. Adhesion is enhanced. (f) The double mutation is present on each side. The salt bridge cannot form to support strand exchange so there is no adhesion.

form. Insertion of Trp2 into its hydrophobic acceptor pocket is stabilized by the salt bridge between Glu89 and the N-terminus. By analogy with other 3D domain swap systems (Bergdoll et al., 1997), it is possible that the hinge loop at the base of the βA strand may be under spring-like tension, perhaps imposed by Pro 6 and neighbouring amino acids. For strand exchange to occur in the strand-swapped dimer (Fig. 5b), an activation barrier must be surmounted as Trp2 leaves the hydrophobic pocket (Haussinger et al., 2004). A transition state in which Trp2 is un-docked is sampled from both sides and is depicted in the interface as an 'unshaded' Trp. Adhesion between the two domains is moderate. The difference in free energy between the 'closed' monomer in which Trp2 is docked into its own domain and the strand-swapped dimer is small because the βA strand enjoys the same contacts in each, the two forms differing only in the angle adopted by the hinge loop. Extension of the N-terminus on one side destabilises both intramolecular and intermolecular docking of Trp2 on that strand (Fig. 5c). Mutual strand exchange between the two domains cannot take place. Cross docking of Trp2 on the wild-type strand is possible but this competes unfavourably with intramolecular docking into its own wild-type domain. Thus, adhesion is very weak. Similar logic applies where the Glu89Ala mutation prevents intramolecular docking on that side. Again, strand exchange cannot be mutual (Fig. 5d). The strand from the Glu89Ala mutant can cross to the wild-type domain but cross docking competes with intramolecular docking of Trp2 on the wild-type side. When the two mutations are in opposing cadherin molecules, they form a complementary pair (Fig. 5e). Here the activation barrier for strand donation by the Glu89Ala mutant is greatly reduced because the salt bridge can form only in the trans position. Intramolecular docking on either side is denied, so cross docking of Trp2 into the pocket of the Gly-Gly mutant is unimpaired. Even though only one strand is exchanged,

adhesion is enhanced compared with the wild-type interaction. Finally, when the double mutation is present in both partners (Fig. 5f), the salt bridge is prevented on either side and adhesion is completely lost.

Removing Trp2 or blocking the hydrophobic pocket

In the experiments described so far, disruption of the salt bridge prevented intramolecular docking of Trp2 in one or both components of the dimer. In an alternative strategy to prevent intramolecular docking, we removed Trp2 by introducing the mutation Trp2Gly or blocked the hydrophobic acceptor pocket with an isoleucine side chain projecting into the cavity using the mutation Ala80Ile. N-cadherin Fc fusion proteins with these mutations were then tested against our panel of K562 transfectants (Fig. 6a). In keeping with the explanation given in Fig. 5, the Trp2Gly mutant acted as a strand acceptor and therefore adhered strongly to the Glu89Ala mutant whereas the Ala80Ile mutant was a strand donor and adhered to the Gly-Gly extension mutant. To determine whether the Trp2Gly mutant and the Ala80Ile mutant adhered strongly to one another, as would be predicted, the proteins were coated separately to Dynabeads and the two types were then mixed and tested for cadherin-dependent aggregation. The mixed preparation of beads formed large clumps, aggregating more strongly than beads coated with wild-type N-cadherin (Fig. 6b). In contrast, beads coated with the Trp2Gly or Ala80Ile mutants and tested separately clustered in twos and threes, whereas beads coated with the negative control mutant, Asp134Ala, were entirely monodisperse.

Discussion

The present report demonstrates decisively that the strand exchange mechanism of cadherin adhesion requires the

formation of a salt bridge between opposing cadherin molecules involving the N-terminus of one molecule and Glu89 of the other. The bond is a major feature of the free energy landscape that governs strand exchange. A second factor is the hydrophobic interaction between Trp2 and its acceptor pocket. For stable adhesion, the energy contributions of both factors are required. In addition, a hydrogen bond formed between the amide nitrogen of Val3 and the carbonyl oxygen of residue 25 may also contribute to the stability of Trp2 intercalation (Haussinger et al., 2004). Formation of the cadherin adhesive dimer can be regarded as a relatively uncomplicated example of the 3D domain swap mechanism for protein oligomerization (Bennett and Eisenberg, 2004; Rousseau et al., 2003). A high-energy barrier must be

Fig. 6. Removing Trp2 or blocking the hydrophobic acceptor pocket. (a) K562 cells expressing wild-type or mutant N-cadherin were tested for adhesion to N-cadherin Fc (1 μg/ml) bearing either the mutation Trp2Gly (W2G) or the pocket-blocking mutation Ala80Ile (A80I). The Trp2Gly mutant acted as a strand acceptor and therefore adhered to cells expressing the Glu89Ala (E89A) mutation. In contrast, the Ala80Ile mutant behaved as a strand donor because intramolecular docking of Trp2 was denied and therefore bound to cells expressing the Gly-Gly N-terminal extension mutant (GG). (b) Dynabeads coated with the Trp2Gly mutant or the Ala80Ile mutant were tested for aggregation separately or as a mixture and compared with aggregation mediated by wild-type N-cadherin. Aggregation was assessed by microscopy. The Asp134Ala (D134A) mutant served as a negative control and here, the 2.8 μm diameter beads remained monodisperse.

overcome as the structural component to be exchanged is released from its own domain and becomes available for exchange, but the difference in free energy between the monomer and the dimer is small. In our experiments, intramolecular docking of Trp2 was prevented in both components of the cadherin dimer by disrupting the salt bridge. This greatly reduced the energy barrier for strand exchange. By using a complementary pair of mutations, the Gly-Gly N-terminal extension on one side and Glu89Ala on the other, the energetics were changed strongly to favour exchange of one strand. These mutants behaved as 'molecular Velcro' forming a strongly adhesive complementary pair but neither adhering to its own kind. We assume that the effect of the Gly-Gly extension in this context was solely to displace the N-terminus away from the acidic side chain of Glu89 to prevent formation of the salt bridge. The assumption is likely to be valid because alternative short extensions to the N-terminus, e.g. Met-Asp-Pro or a single Cys, were observed to have a similar complementary effect with the Glu89Ala mutant (data not shown). Strong adhesion was observed even with unprocessed N-cadherin, though titrations suggest that the prodomain had an inhibitory effect compared with an extension of only two amino acids. Some steric hindrance by the prodomain would be expected because the long flexible linker (Koch et al., 2004) would allow freedom of movement of the uncleaved domain to interfere with the strand exchange process.

By analogy with other 3D domain swap systems, it is likely that the activation energy for strand exchange can be reduced by means other than mutating the Glu89 salt bridge or blocking the hydrophobic acceptor pocket. In other proteins, altering the length of the hinge loop or relieving backbone tension imposed by proline residues in this region has increased the propensity to dimerize by many orders of magnitude (Green et al., 1995; Rousseau et al., 2003; Rousseau et al., 2001). It is not known whether similar changes to the βA strand in cadherins would modulate intramolecular docking of Trp2 and alter the free energy balance in favour of strand exchange.

Our results were obtained with N-cadherin, a classical cadherin. But multiple alignment of amino acid sequences of non-classical cadherins and structural modelling by our laboratory and others (May et al., 2005) suggests that non-classical (Type II) cadherins, desmocollins and desmogleins all have a similar strand exchange mechanism dependent on a salt bridge in the position described here. In the protocadherin family, N-terminal peptide analysis suggests that protocadherins α also have a conserved tryptophan as the second amino acid (Gevaert et al., 2003), indicating that the same mechanism may apply in this group also. It is possible that variations of the strand swap model apply throughout the whole cadherin family.

Three important issues remain unresolved by the present report. First, the controversial matter of cadherin type-specificity. Conclusions about specificity have been subject to the particular tests used and the pairs of cadherins investigated (Foty and Steinberg, 2005; Klingelhofer et al., 2000; Niessen and Gumbiner, 2002; Nose et al., 1990). In cell aggregation assays, cells usually segregate according to cadherin type. This behaviour has been attributed to structural features in domain 1 (Nose et al., 1990) or, alternatively, to subtle quantitative differences in the level of cell surface cadherin expressed by different cell types in the mixed population (Foty and
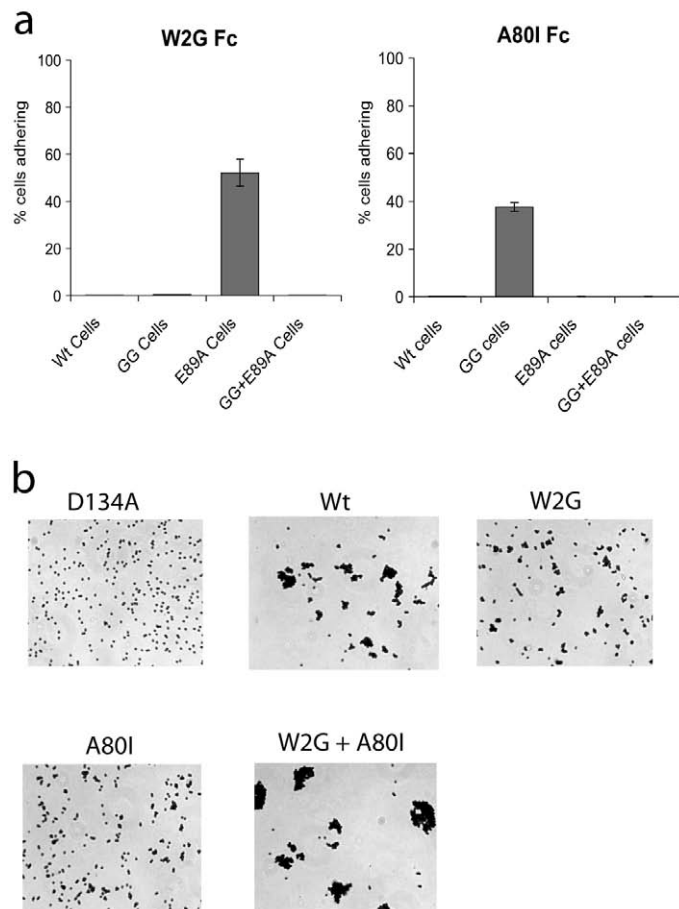
Steinberg, 2005). Experiments in our laboratory (not shown) have demonstrated clear homophilic preference by N- and E-cadherins in the adhesion assay described here. To explain specificity by the strand exchange mechanism, we reasoned that optimal adhesion between wild-type cadherins may require free energy changes accompanying mutual strand exchange to be equal on both sides of the adhesive dimer. At least two factors influence the energy landscape, the N-terminal salt bridge and the hydrophobic interaction between Trp2 and its pocket. The former would be affected by the electrostatic environment in the vicinity of Glu89, and the latter by non-conserved amino acids lining the hydrophobic pocket. Our preliminary experiments, so far, are consistent with the hypothesis of energy balance and implicate both factors in the specificity displayed by N- and E-cadherins. A second question concerns the inhibitory effects of peptides that contain the motif HAV, which is conserved in classical cadherins, or related motifs present in other cadherins (Makagiansar et al., 2001; Noe et al., 1999; Runswick et al., 2001; Williams et al., 2000). The HAV motif is not essential for adhesion (Renaud-Young and Gallin, 2002) and the mechanism of action of these peptides is unknown. Our experiments do not enlighten the issue. HAV peptides may block other putative interaction sites in domain 1 observed in crystal structures (Boggon et al., 2002; Shapiro et al., 1995) or, perhaps, cause local conformational changes that interfere with strand exchange. Finally, it is still unclear whether cadherin adhesion involves domains 1 and 2 only (Shan et al., 2004) or whether contacts involving inner domains also play a role (Chappuis-Flament et al., 2001; Perret et al., 2004; Zhu et al., 2003). The second domain, EC2, is required for correct coordination of calcium in the junction between EC1 and EC2 and the disruptive effect of the Asp134Ala mutation in the present experiments demonstrates that, in this respect, EC2 is essential for strand exchange. But our results do not rule out the possibility that EC3 or EC4 could provide additional contact sites or be involved in other ways. It is important to emphasise that assays used in different studies to test for adhesive contacts involving inner domains have varied greatly in sensitivity and results must be interpreted accordingly. The cell adhesion test in the present report is very robust and is unlikely to reveal weak interactions. In contrast, our bead aggregation assay is more sensitive and it is notable that the Trp2Gly mutant and the Ala80Ile mutant which, individually, could not undergo strand exchange by tryptophan docking showed weak but detectable aggregation when tested separately. The result may reflect the presence of one or more additional contact sites. It is consistent with very weak adhesion seen in other circumstances where strand exchange cannot occur, for example the interaction between E-cadherin lacking domain 1 and the wild-type molecule (Renaud-Young and Gallin, 2002). It is pertinent to observe that formation of the salt bridge reported here is likely to require correct angular alignment of opposing N-terminal domains. The presence of the inner domains may facilitate optimal orientation; indeed, the curvature of the complete extracellular region (Boggon et al., 2002) could be significant in this respect.

The present report offers new insights into the strand exchange mechanism. The observation that cadherin affinity can be greatly increased by lowering activation energy using salt bridge mutations provides a rational basis for designing alternative strategies for modulating cadherin adhesion.

References

Baumgartner, W., Hinterdorfer, P., Ness, W., Raab, A., Vestweber, D., Schindler, H. and Drenckhahn, D. (2000). Cadherin interaction probed by atomic force microscopy. *Proc. Natl. Acad. Sci. USA* **97**, 4005-4010.

Bennett, M. J. and Eisenberg, D. (2004). The evolving role of 3D domain swapping in proteins. *Structure (Camb.)* **12**, 1339-1341.

Bergdoll, M., Remy, M. H., Cagnon, C., Masson, J. M. and Dumas, P. (1997). Proline-dependent oligomerization with arm exchange. *Structure* **5**, 391-401.

Boggon, T. J., Murray, J., Chappuis-Flament, S., Wong, E., Gumbiner, B. M. and Shapiro, L. (2002). C-cadherin ectodomain structure and implications for cell adhesion mechanisms. *Science* **296**, 1308-1313.

Cavallaro, U. and Christofori, G. (2004). Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat. Rev. Cancer* **4**, 118-132.

Chappuis-Flament, S., Wong, E., Hicks, L. D., Kay, C. M. and Gumbiner, B. M. (2001). Multiple cadherin extracellular repeats mediate homophilic binding and adhesion. *J. Cell Biol.* **154**, 231-243.

Corps, E., Carter, C., Karecla, P., Ahrens, T., Evans, P. and Kilshaw, P. (2001). Recognition of E-cadherin by integrin alpha(E)beta(7): requirement for cadherin dimerization and implications for cadherin and integrin function. *J. Biol. Chem.* **276**, 30862-30870.

Foty, R. A. and Steinberg, M. S. (2005). The differential adhesion hypothesis: a direct evaluation. *Dev. Biol.* **278**, 255-263.

Frank, M. and Kemler, R. (2002). Protocadherins. *Curr. Opin. Cell Biol.* **14**, 557-562.

Gevaert, K., Goethals, M., Martens, L., Van Damme, J., Staes, A., Thomas, G. R. and Vandekerckhove, J. (2003). Exploring proteomes and analyzing protein processing by mass spectrometric identification of sorted N-terminal peptides. *Nat. Biotechnol.* **21**, 566-569.

Green, S. M., Gittis, A. G., Meeker, A. K. and Lattman, E. E. (1995). One-step evolution of a dimer from a monomeric protein. *Nat. Struct. Biol.* **2**, 746-751.

Harrison, O. J., Corps, E. M., Berge, T. and Kilshaw, P. J. (2005). The mechanism of cell adhesion by classical cadherins: the role of domain 1. *J. Cell Sci.* **118**, 711-721.

Haussinger, D., Ahrens, T., Sass, H. J., Pertz, O., Engel, J. and Grzesiek, S. (2002). Calcium-dependent homoassociation of E-cadherin by NMR spectroscopy: changes in mobility, conformation and mapping of contact regions. *J. Mol. Biol.* **324**, 823-839.

Haussinger, D., Ahrens, T., Aberle, T., Engel, J., Stetefeld, J. and Grzesiek, S. (2004). Proteolytic E-cadherin activation followed by solution NMR and X-ray crystallography. *EMBO J.* **23**, 1699-1708.

Hirano, S., Suzuki, S. T. and Redies, C. (2003). The cadherin superfamily in neural development: diversity, function and interaction with other molecules. *Front. Biosci.* **8**, d306-d355.

Kitagawa, M., Natori, M., Murase, S., Hirano, S., Taketani, S. and Suzuki, S. T. (2000). Mutation analysis of cadherin-4 reveals amino acid residues of EC1 important for the structure and function. *Biochem. Biophys. Res. Commun.* **271**, 358-363.

Klingelhofer, J., Troyanovsky, R. B., Laur, O. Y. and Troyanovsky, S. (2000). Amino-terminal domain of classic cadherins determines the specificity of the adhesive interactions. *J. Cell Sci.* **113**, 2829-2836.

Koch, A. W., Farooq, A., Shan, W., Zeng, L., Colman, D. R. and Zhou, M. M. (2004). Structure of the neural (N-) cadherin prodomain reveals a cadherin extracellular domain-like fold without adhesive characteristics. *Structure (Camb.)* **12**, 793-805.

Liu, Y. and Eisenberg, D. (2002). 3D domain swapping: as domains continue to swap. *Protein Sci.* **11**, 1285-1299.

Makagiansar, I. T., Avery, M., Hu, Y., Audus, K. L. and Siahaan, T. J. (2001). Improving the selectivity of HAV-peptides in modulating E-cadherin-E-cadherin interactions in the intercellular junction of MDCK cell monolayers. *Pharm. Res.* **18**, 446-453.

May, C., Doody, J. F., Abdullah, R., Balderes, P., Xu, X., Chen, C. P., Zhu, Z., Shapiro, L., Kussie, P., Hicklin, D. J. et al. (2005). Identification of a transiently exposed VE-cadherin epitope that allows for specific targeting of an antibody to the tumor neovasculature. *Blood* **105**, 4337-4344.

Niessen, C. M. and Gumbiner, B. M. (2002). Cadherin-mediated cell sorting

not determined by binding or adhesion specificity. *J. Cell Biol.* **156**, 389-399.

Noe, V., Willems, J., Vandekerckhove, J., Roy, F. V., Bruyneel, E. and Mareel, M. (1999). Inhibition of adhesion and induction of epithelial cell invasion by HAV-containing E-cadherin-specific peptides. *J. Cell Sci.* **112**, 127-135.

Nollet, F., Kools, P. and van Roy, F. (2000). Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *J. Mol. Biol.* **299**, 551-572.

Nose, A., Tsuji, K. and Takeichi, M. (1990). Localization of specificity determining sites in cadherin cell adhesion molecules. *Cell* **61**, 147-155.

Ozawa, M. and Kemler, R. (1990). Correct proteolytic cleavage is required for the cell adhesive function of uvomorulin. *J. Cell Biol.* **111**, 1645-1650.

Ozawa, M., Engel, J. and Kemler, R. (1990). Single amino acid substitutions in one Ca2+ binding site of uvomorulin abolish the adhesive function. *Cell* **63**, 1033-1038.

Perret, E., Leung, A., Feracci, H. and Evans, E. (2004). Trans-bonded pairs of E-cadherin exhibit a remarkable hierarchy of mechanical strengths. *Proc. Natl. Acad. Sci. USA* **101**, 16472-16477.

Pertz, O., Bozic, D., Koch, A. W., Fauser, C., Brancaccio, A. and Engel, J. (1999). A new crystal structure, Ca2+ dependence and mutational analysis reveal molecular details of E-cadherin homoassociation. *EMBO J.* **18**, 1738-1747.

Renaud-Young, M. and Gallin, W. J. (2002). In the first extracellular domain of E-cadherin, heterophilic interactions, but not the conserved His-Ala-Val motif, are required for adhesion. *J. Biol. Chem.* **277**, 39609-39616.

Rousseau, F., Schymkowitz, J. W., Wilkinson, H. R. and Itzhaki, L. S. (2001). Three-dimensional domain swapping in p13suc1 occurs in the unfolded state and is controlled by conserved proline residues. *Proc. Natl. Acad. Sci. USA* **98**, 5596-5601.

Rousseau, F., Schymkowitz, J. W. and Itzhaki, L. S. (2003). The unfolding story of three-dimensional domain swapping. *Structure (Camb.)* **11**, 243-251.

Runswick, S. K., O'Hare, M. J., Jones, L., Streuli, C. H. and Garrod, D. R. (2001). Desmosomal adhesion regulates epithelial morphogenesis and cell positioning. *Nat. Cell. Biol.* **3**, 823-830.

Schubert, W. D., Urbanke, C., Ziehm, T., Beier, V., Machner, M. P., Domann, E., Wehland, J., Chakraborty, T. and Heinz, D. W. (2002). Structure of internalin, a major invasion protein of Listeria monocytogenes, in complex with its human receptor E-cadherin. *Cell* **111**, 825-836.

Shan, W., Yagita, Y., Wang, Z., Koch, A., Svenningsen, A. F., Gruzglin, E., Pedraza, L. and Colman, D. R. (2004). The minimal essential unit for cadherin-mediated intercellular adhesion comprises extracellular domains 1 and 2. *J. Biol. Chem.* **279**, 55914-55923.

Shapiro, L., Fannon, A. M., Kwong, P. D., Thompson, A., Lehmann, M. S., Grubel, G., Legrand, J. F., Als-Nielsen, J., Colman, D. R. and Hendrickson, W. A. (1995). Structural basis of cell-cell adhesion by cadherins. *Nature* **374**, 327-337.

Tamura, K., Shan, W. S., Hendrickson, W. A., Colman, D. R. and Shapiro, L. (1998). Structure-function analysis of cell adhesion by neural (N-) cadherin. *Neuron* **20**, 1153-1163.

Thiery, J. P. (2003). Cell adhesion in development: a complex signaling network. *Curr. Opin. Genet. Dev.* **13**, 365-371.

Wheelock, M. J. and Johnson, K. R. (2003). Cadherins as modulators of cellular phenotype. *Annu. Rev. Cell Dev. Biol.* **19**, 207-235.

Williams, E., Williams, G., Gour, B. J., Blaschuk, O. W. and Doherty, P. (2000). A novel family of cyclic peptide antagonists suggests that N-cadherin specificity is determined by amino acids that flank the HAV motif. *J. Biol. Chem.* **275**, 4007-4012.

Yang, S., Cho, S. S., Levy, Y., Cheung, M. S., Levine, H., Wolynes, P. G. and Onuchic, J. N. (2004). Domain swapping is a consequence of minimal frustration. *Proc. Natl. Acad. Sci. USA* **101**, 13786-13791.

Zhu, B., Chappuis-Flament, S., Wong, E., Jensen, I. E., Gumbiner, B. M. and Leckband, D. (2003). Functional analysis of the structural basis of homophilic cadherin adhesion. *Biophys. J.* **84**, 4033-4042.