

Dynamic and Static Interactions between p120 Catenin and E-Cadherin Regulate the Stability of Cell-Cell Adhesion

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SUMMARY

The association of p120 catenin (p120) with the juxtamembrane domain (JMD) of the cadherin cytoplasmic tail is critical for the surface stability of cadherin-catenin cell-cell adhesion complexes. Here, we present the crystal structure of p120 isoform 4A in complex with the JMD core region (JMD_{core}) of E-cadherin. The p120 armadillo repeat domain contains modular binding pockets that are complementary to electrostatic and hydrophobic properties of the JMD_{core}. Single-residue mutations within the JMD_{core}-binding site of p120 abolished its interaction with E- and N-cadherins *in vitro* and in cultured cells. These mutations of p120 enabled us to clearly differentiate between N-cadherin-dependent and -independent steps of neuronal dendritic spine morphogenesis crucial for synapse development. NMR studies revealed that p120 regulates the stability of cadherin-mediated cell-cell adhesion by associating with the majority of the JMD, including residues implicated in clathrin-mediated endocytosis and Hakai-dependent ubiquitination of E-cadherin, through its discrete “dynamic” and “static” binding sites.

INTRODUCTION

p120 catenin (p120) is an armadillo (ARM) repeat-containing protein that, along with the classical cadherins, β -catenin and α -catenin, plays a crucial role in the regulation of cell-cell adhesion at adherens junctions (AJs) (Daniel and Reynolds, 1995; Nishimura and Takeichi, 2009; Reynolds et al., 1992, 1994). The classical cadherins mediate Ca²⁺-dependent homophilic adhesion via *trans*-dimerization of cadherin ectodomains between adjacent cells (Shapiro and Weis, 2009) and by having the cadherin cytoplasmic tail participate in cytoskeleton reorga-

nization and intracellular signaling through catenins (Nishimura and Takeichi, 2009; Yamada and Nelson, 2007). β -catenin associates with the catenin-binding domain (CBD) and functionally links cadherins with the actin cytoskeleton through α -catenin (Yamada et al., 2005), while p120 is responsible for stabilizing cadherin-catenin complexes at the cell surface by interacting with the juxtamembrane domain (JMD; Figure 1A) (Davis et al., 2003; Ireton et al., 2002; Lampugnani et al., 1997; Thoreson et al., 2000; Xiao et al., 2003). The cadherin JMD has been implicated in various processes, including cadherin clustering and adhesive strengthening (Yap et al., 1998), promotion of axon outgrowth (Riehl et al., 1996), and suppression of cell motility (Chen et al., 1997).

Previous studies have shown that uncoupling of the p120-JMD interaction or a reduction in p120 protein levels in cultured cells significantly increases levels of cadherin internalization and reduces the amount of cadherin available for cell-cell adhesion (Davis et al., 2003; Ireton et al., 2002; Miyashita and Ozawa, 2007b; Perez-Moreno et al., 2006; Xiao et al., 2003), thereby implicating p120 as a gatekeeper of cadherin turnover in vertebrates (Peifer and Yap, 2003). The significance of the p120-JMD interaction is underscored by the fact that the JMD is highly conserved among classical (type I), type II, and some invertebrate cadherins (Nollet et al., 2000). Prior work by Thoreson et al. (2000) has determined that the JMD core region (JMD_{core}; residues 758–775 in mouse E-cadherin; Figure 1A) is crucial for the p120-JMD interaction. Conversely, p120 associates with the cadherin JMD through a central ARM repeat domain (ARM domain; Figure 1B) (Daniel and Reynolds, 1995). The p120 ARM domain is well conserved from fly to human (>40% primary sequence identity [Myster et al., 2003]) and is flanked by an N-terminal regulatory region (NTR) and a C-terminal tail region (CTR).

The loss of E-cadherin expression, a major hallmark of tumor malignancy (Hanahan and Weinberg, 2000), is induced by a variety of factors, including transcriptional regulation, mutation, and aberrant cadherin internalization (Mosesson et al., 2008). Consistent with the critical role of p120 in the regulation of cadherin turnover, the loss, downregulation, or mislocalization of p120 in tumors has been linked to poor prognoses

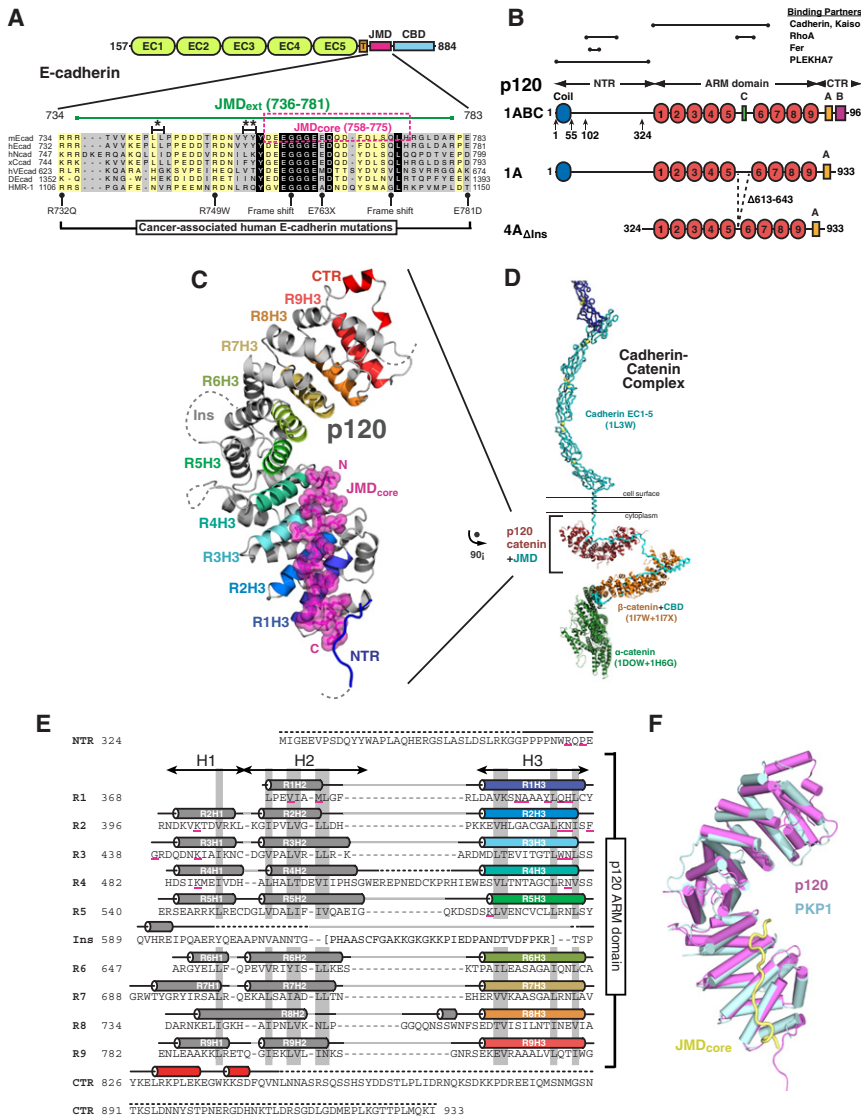


Figure 1. E-Cadherin JMD_{core} Interacts with p120 ARM Repeats 1–5

(A) Schematic representation of E-cadherin. E-cadherin consists of the extracellular cadherin domains 1–5 (EC1–5), the transmembrane region (T) and the cytoplasmic tail, which contains the JMD and CBD. The JMD_{ext} and the JMD_{core} used in this study are indicated. Multiple sequence alignment of seven cadherin JMD sequences is shown (mEcad, mouse E-cadherin; hEcad, human E-cadherin; hNcad, human N-cadherin; xCcad, frog C-cadherin; hVEcad, human VE-cadherin; DEcad, fly DE-cadherin; HMR-1, nematode HMR-1). Identical residues are highlighted in black boxes, and residues conserved in at least four cadherins are shown in yellow. * indicates the endocytic LL motif. ** indicates tyrosine phosphorylation sites.

(B) Schematic representation of p120. p120 consists of the central ARM domain (ARM repeats are indicated in red) flanked by the NTR and CTR. Various p120 isoforms result from four transcription start sites (residue 1, 55, 102, 324) and three alternatively spliced exons (A, B, and C). Isoforms 1ABC and 1A contain a coiled-coil domain (Coil) within the NTR. p120-4A_{ΔIns} was created by deletion of residues 613–643 in the insert region of isoform 4A. Binding partners of p120 and their binding sites are shown (Cadherin [Daniel and Reynolds, 1995; Ireton et al., 2002], Kaiso [Daniel and Reynolds, 1999], RhoA [Castaño et al., 2007; Yanagisawa et al., 2008], PLEKHA7 [Meng et al., 2008], and Fer kinase [Lee et al., 2008; Xu et al., 2004]).

(C) Crystal structure of the p120-4A_{ΔIns}/JMD_{core} complex. p120 contains nine ARM repeats (R1–9) with each repeat consisting of three α helices (H1, H2, and H3), except for R1 and R8 (H3 helices from ARM R1–9 are shown in different colors). The modified insert region (Ins), loops between R4H2 and R4H3, NTR, and CTR are partially disordered (dashed lines). The JMD_{core} is shown in magenta (stick and space filling representation). See Table S1 for structure determination and refinement statistics. The “head-to-tail” intercomplex contact site observed in both crystal forms is shown in Figure S1.

(D) Model of the cadherin-catenin complex.

(E) Primary sequence and secondary structure of p120-4A_{ΔIns}. The primary sequence of the ARM domain is aligned according to H1–3 helices to highlight the sequence conservation (residues shaded gray). Disordered regions are shown as dashed lines, and gaps are indicated by light gray line above the sequence. Square brackets indicate the deleted residues from the insert region (residues 613–643). The JMD_{core}-binding site residues are indicated by magenta underscore.

(F) Superposition of the p120-4A_{ΔIns}/JMD_{core} complex and PKP1 ARM domain (PDB code 1XM9).

(Thoreson and Reynolds, 2002). The major route of E-cadherin internalization appears to be clathrin-mediated endocytosis involving a noncanonical endocytic dileucine motif (LL motif) (Miyashita and Ozawa, 2007a, 2007b). In addition, ubiquitination-dependent endocytosis of E-cadherin induced by the E3 ubiquitin ligase Hakai (Fujita et al., 2002) and presenilin-1/ γ -secretase-mediated cleavage of E-cadherin (Marambaud et al., 2002) have been implicated in the depletion of E-cadherin from the cell surface. As the endocytic LL motif (residues 743–744) and tyrosine phosphorylation sites (residues 755–756) involved in recruitment of Hakai are both located near the JMD_{core} (Figure 1A), the association of p120 with the JMD is proposed to sterically hinder endocytic machinery and Hakai from associ-

ating with cadherin (Fujita et al., 2002; Miyashita and Ozawa, 2007b). However, the mechanisms through which p120 regulates cadherin endocytosis have remained elusive because of a lack of detailed knowledge of the p120-JMD interaction. Moreover, a novel missense germline mutation within the JMD of human E-cadherin (R749W, Arg751 in mouse; Figure 1A) linked to hereditary diffuse gastric cancer (HDGC) was recently discovered and shown to have impaired cell adhesive function in vitro (Kaurah et al., 2007). This raises the possibility that such mutations might disrupt the p120-E-cadherin interaction, thereby contributing to an increased risk for diffuse gastric cancer.

In the present study, we have determined the crystal structure of p120 in complex with the JMD_{core} peptide at 2.4 Å resolution.

Structure-based mutagenesis studies in both cultured epithelial and neuronal cells demonstrated that single-residue mutations within the JMD_{core}-binding site of p120 is sufficient to uncouple p120 from E- and N-cadherins and to delineate multifunctional roles of p120 in cell-cell adhesion and other signaling processes. Nuclear magnetic resonance (NMR) data revealed that p120 regulates the stability of cadherin-mediated cell-cell adhesion by associating with the majority of the E-cadherin JMD, including the endocytic LL motif and tyrosine-phosphorylation sites, through discrete “dynamic” and “static” binding sites. In addition, we propose a model for a p120-dependent cadherin clustering mechanism, which involves the JMD-induced oligomerization of p120.

RESULTS AND DISCUSSION

Overall Structure of the p120-4A_{ΔIns}/JMD_{core} Complex

To better understand how the interaction between p120 and the JMD modulates the stability of cadherin in cell-cell adhesion, we subjected variants of p120 to cocrystallization with JMD fragments. We successfully crystallized a modified form of human p120 isoform 4A (p120-4A_{ΔIns}; Figure 1B) in complex with the mouse E-cadherin JMD_{core} fragment (mouse and human have identical primary sequences; Figure 1A). p120-4A_{ΔIns} was produced by deletion of residues 613–643 from the insert region (residues 589–648), which resulted in noticeably higher protein stability and crystallizability. The three-dimensional structure of p120-4A_{ΔIns}/JMD_{core} complex was determined in two crystal forms at 2.4 Å (form I) and 3.0 Å (form II) resolution (Table S1 available online). The following characterization of the complex is based mainly on form I, as the complex structures were nearly identical in both forms except for an increased disorder in the C terminus of p120 in form II.

The crystal structure of the p120-4A_{ΔIns}/JMD_{core} complex provides the first opportunity to examine the three-dimensional architecture of p120, as well as the binding interface between p120 and the cadherin JMD (Figure 1C). p120-4A_{ΔIns} consists of a central arch-shaped ARM domain (residues 368–825) accompanied by largely disordered NTR (residues 324–367) and CTR (residues 826–933). The bound JMD_{core} peptide is stretched over the N-terminal half of the p120 ARM domain in the opposite orientation (Figure 1C), resembling the orientation of the CBD bound to the ARM domain of β-catenin (Huber and Weis, 2001). The p120 ARM domain is similar to that of plakophilin-1 (PKP1) (Choi and Weis, 2005), as it contains nine ARM repeats (R1–9) and a partially unstructured insert region (Figures 1C and 1E). They can be superposed with a root mean square distance (RMSD) of 1.2 Å over 324 C_α atoms (Figure 1F). While the previously determined PKP1 structure is limited to the ARM domain (Choi and Weis, 2005), p120-4A_{ΔIns} contains unique structural features beyond the ARM domain; the last ten residues of the NTR of p120 wrap over the C terminus of cadherin JMD_{core}, and the first 21 residues of the CTR of p120 form two α helices that fold over the hydrophobic surface of ARM R9 (Figure 1C). Though other isoforms contain longer NTR, CTR, and insert region compared to p120-4A, the structures of ARM domain, NTR, and CTR revealed here are likely conserved in all p120 isoforms.

The p120-4A_{ΔIns}/JMD_{core} complex structure has allowed us to bring together the three-dimensional structures of C-cadherin ectodomain (PDB code 1L3W) (Boggon et al., 2002), the β-catenin/CBD complex (PDB codes 1I7W and 1I7X) (Huber and Weis, 2001), and α-catenin fragments (PDB codes 1DOW and 1H6G) (Pokutta and Weis, 2000; Yang et al., 2001), finally revealing the multimeric arrangement of the cadherin-catenin complex in its entirety (Figure 1D). As these complexes are expected to cluster to mediate cell-cell adhesion (Yap et al., 1998), the presence of JMD-bound p120 (105 Å × 55 Å × 40 Å) in a compact space between the plasma membrane and the β-catenin/CBD complex is likely to restrict other proteins (e.g., endocytic machinery and Hakai) from gaining access to the JMD (Figure 1D). Although JMD residues 734–757 were not accounted for in our p120/JMD complex structure, the C terminus of the JMD_{core} (His775) is only separated by eight residues from the N terminus of the CBD (Val784) from the β-catenin/CBD complex (Huber and Weis, 2001). This places the N terminus of p120 in close proximity to the ARM domain of β-catenin, which is consistent with the role of p120 in recruiting Fer kinase through its NTR to modulate the cadherin-β-catenin interaction (Lee et al., 2008; Xu et al., 2004).

E-Cadherin JMD_{core} Interacts with p120 ARM Repeats 1–5

The p120-JMD_{core} interface buries approximately 2400 Å² of total solvent accessible surface area, and this interface can be further divided into two regions on the basis of different types of intermolecular interactions (Figure 2A). The first region involves extensive electrostatic interactions between the N-terminal acidic region (residues 758–766) of the JMD_{core} and the p120 basic ARM groove formed along the third helix (H3) of ARM R1–5 (Figure 2B). Five salt bridges are formed between acidic residues (Asp758, Glu759, Glu760, Glu764, and Glu765) from the cadherin JMD_{core} and basic residues (Lys401, Lys433, Lys444, Lys486, and Lys574) from p120 (Figure 2C). In addition, the triple glycine motif (Gly761, Gly762, and Gly763) of the JMD_{core} forms a turn that fits into a trough formed by Phe437, Trp477, and Asn478 of p120. The backbone of the JMD_{core} forms hydrogen bonds with p120 residues, Asn434, Asn478, and Asn536 (Figure 2C). Several p120 residues involved in this interface are conserved within p120 ARM R2–7 (Figure 1E): the first turn of H1 helices from ARM R2–5 contain conserved basic residues, including Lys401, Lys444, and Lys486, and the last turn of H3 helices from ARM R2–7 contain conserved asparagines, including Asn434, Asn478, and Asn536. In contrast, the second region of the p120-4A_{ΔIns}/JMD_{core} interface involves mainly hydrophobic interactions between the C-terminal anchor region of the JMD_{core} (residues 767–775) and the N terminus of p120 (Figure 2B). The anchor region of the JMD_{core} forms a single ₃₁₀-helix turn that is wedged between ARM R1 and the NTR of p120 (Figure 2D). Consequently, the side chain of Leu774 from the JMD_{core} is buried or “anchored” into a hydrophobic pocket formed between H2 and H3 helices of ARM R1, involving Pro366, Val371, Met374, Val382, Ala386, and Tyr389 (Figures 2B and 2D). Given that the triple glycine motif with two adjoining glutamates (EGGGE) and the anchoring leucine are strictly conserved in the JMD_{core} from fly DE-cadherin to human

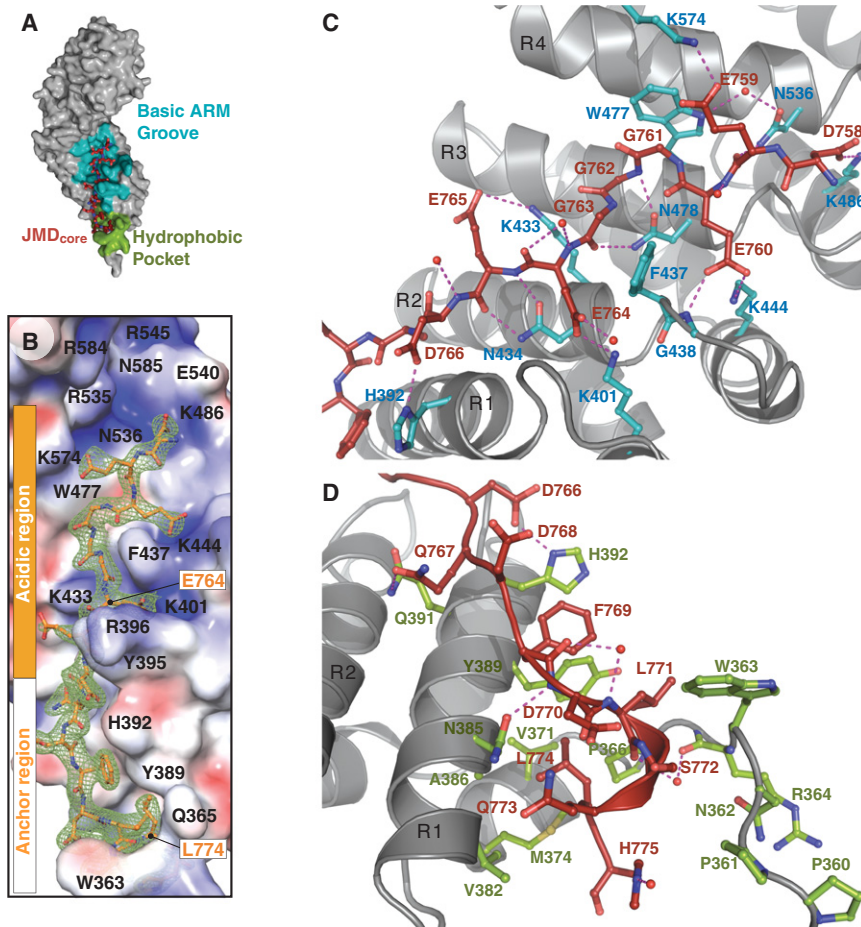


Figure 2. The JMD_{core}-Binding Site Consists of the Basic ARM Groove and Hydrophobic Binding Pocket

(A) Overview of the JMD_{core}-binding site of p120-4A_{ΔIns} (same orientation as Figure 1C). The JMD_{core} (red) is bound to the basic ARM groove (blue) and the N-terminal hydrophobic pocket (green).

(B) The surface electrostatic potential of the JMD_{core}-binding site of p120 with positively and negatively charged regions in blue and red, respectively. 2F_o-F_c electron density map (green mesh; contoured at $\sigma_A = 1.5$) of the JMD_{core} (orange) is shown.

(C) The electrostatic interface between p120 and the JMD_{core}. The JMD_{core} N-terminal acidic region (red; residues 758–766) forms extensive electrostatic interaction with the p120 basic ARM groove (blue). Red spheres represent water molecules.

(D) The hydrophobic interface between p120 and the JMD_{core}. The side chain of Leu774 from the C-terminal anchor region of JMD_{core} (red; residues 767–775) is buried into the p120 hydrophobic pocket. Deletion of residues 613–643 did not cause any detrimental effect on the structure of p120 or the interaction between p120 and the E-cadherin JMD (see Figure S2).

E-cadherin (Figure 1A), we propose that both electrostatic and hydrophobic interactions are equally important for the p120-JMD_{core} interaction.

Aside from interactions within the complex, several residues from the anchor region of the JMD_{core} and the NTR of p120 are involved in intercomplex association with the ARM domain from an adjacent p120 molecule in the crystal (Figure S1). Intriguingly, in both crystal forms, p120-4A_{ΔIns}/JMD_{core} complexes assemble into head-to-tail oligomers through this interface, and its periodicity (~60 Å) is similar to the spacing observed in the ordered array of cadherins along the desmosomal midline of human epidermis (Al-Amoudi et al., 2007). Certainly, one must exercise caution when distinguishing protein-protein contacts observed in the crystal to be specific or from a packing contact, and, moreover, neither p120 nor the cadherin cytoplasmic tail alone self-associates in yeast two-hybrid and blot overlay assays, respectively (Daniel and Reynolds, 1995; Yap et al., 1998). We thus employed other approaches to examine the potential JMD-induced oligomerization of p120, including dynamic light-scattering and chemical crosslinking analyses of p120 wild-type (WT) and a W363A mutant (which should compromise the intercomplex interface). Unfortunately, these experiments were hampered by the aggregation-prone nature of p120. Analytical ultracentrifugation analysis of p120 at a low protein concentration (≤ 1.0 mg/ml), however, indicated that

both p120 WT and W363A are mostly “monomeric” in the presence of JMD peptide (data not shown). Nonetheless, the JMD-induced oligomerization of p120 observed in the crystals provides a basis for further examining a possible model for cadherin clustering, a phenomenon presumably facilitated by the cadherin JMD and p120 during maturation of cell-cell junctions (Anastasiadis and Reynolds, 2000; Thoreson et al., 2000; Yap et al., 1997; Yap et al., 1998).

p120 Residues Lys401 and Asn478 Are Essential for Interaction with E-Cadherin

To confirm the significance of the crystallographically determined JMD-binding site of p120, we performed a pulldown assay with glutathione S-transferase-fused JMD residues 747–781 (GST-JMD) and p120-4A_{ΔIns} containing single-residue mutations within the JMD_{core}-binding site (K401M, K444M, W477A, or N478A) or a mutation (W363A) in the NTR that is not expected to affect the p120-JMD interaction (Figure 3A). Circular dichroism (CD) analyses show that the structural integrity of p120-4A_{ΔIns} was not affected by the partial deletion of the insert region or by the single-residue mutations (Figure S2A). Tight association of p120-4A_{ΔIns} WT with GST-JMD (Figure 3A), equivalent to that observed for p120-4A (Figure S2B), indicates that residues 613–643, which contain a RhoGTPase-binding site (residues 622–628) (Yanagisawa et al., 2008), are not required for the interaction between p120 and the JMD. In contrast, alteration of the trough structure (W477A or N478A) or the elimination of a basic residue (K401M or K444M) in the p120 ARM groove was sufficient to disrupt the interaction, though some residual

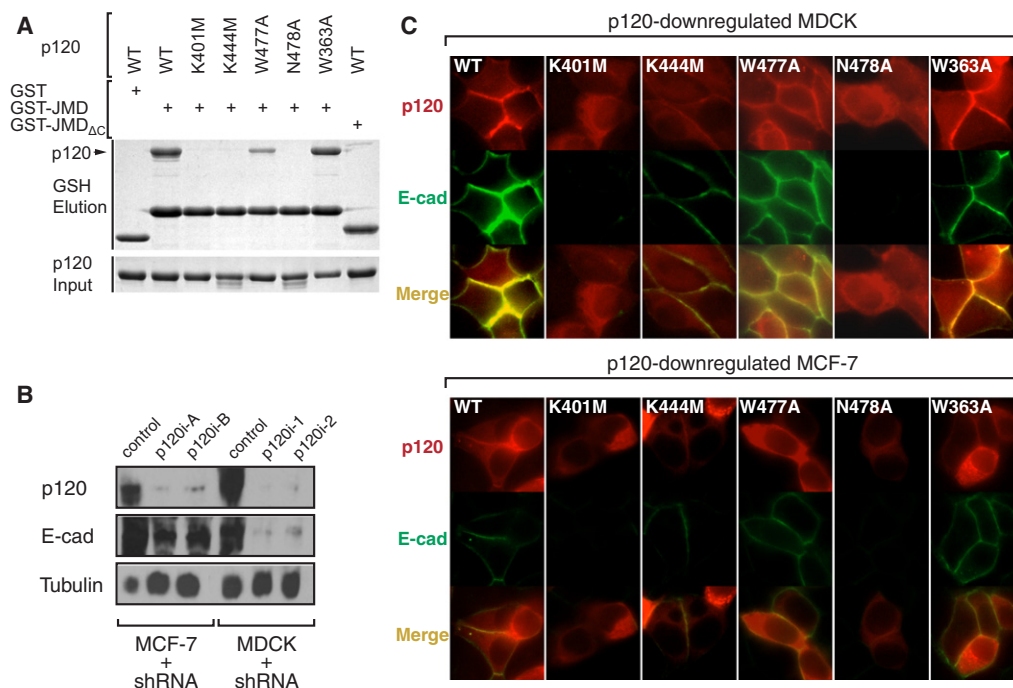


Figure 3. p120 Residues Lys401 and Asn478 Are Necessary for the Colocalization of p120 with E-Cadherin at AJs

(A) GST-JMD pulldown assay with p120-4A_{ΔIns}. GST-JMD (residues 747–781), GST-JMD_{ΔC} (residues 747–775), or GST was used to perform pulldown assay with p120-4A_{ΔIns} WT or mutants (K401M, K444M, W477A, N478A, or W363A). W363A mutation did not affect the p120-JMD interaction since the side chain of Trp363 is not part of the JMD-binding site.

(B) Lysates from MCF-7 or MDCK cells stably expressing shRNA (controls transfected with scrambled shRNA; p120i-A and p120i-B are p120-downregulated monoclonal cell lines in MCF-7; p120i-1 and p120i-2 are p120-downregulated monoclonal cell lines in MDCK) were analyzed by western blotting for levels of p120 and E-cadherin (E-cad). Tubulin levels were used as a loading control.

(C) p120-downregulated MCF-7 cells (p120i-A) or p120-downregulated MDCK cells (p120i-1) were transfected with a mammalian expression vector encoding for p120-1A-mRFP WT or mutants (same as in A).

See also Figure S3 for the expression of p120-1A-mRFP WT or mutants in MDCK and MCF-7 cells expressing endogenous p120.

association was observed with the W477A mutant. In addition, deletion of the C-terminal JMD residues 776–781 located outside of the JMD_{core} also abolished the interaction. These observations confirm that the electrostatic interface between the p120 basic ARM groove and the acidic region of the JMD_{core} are crucial for the p120-JMD interaction and reveal that tight association between p120 and the JMD requires a series of binding pockets that include additional interactions outside of the core region.

To further analyze the JMD_{core}-binding site of p120 in the context of cell-cell adhesion, we tested the ability of these p120 mutants to colocalize with E-cadherin at AJs by transiently expressing p120 isoform 1A (Figure 1B) fused to a monomeric red fluorescent protein (p120-1A-mRFP) in p120-downregulated Madin-Darby canine kidney (MDCK) and MCF-7 cells (Figure 3B). In both cell types, the p120 WT as well as the K444M and W477A mutants colocalized with endogenous E-cadherin at AJs (Figure 3C). The observed association of the K444M mutant with the E-cadherin JMD in cells appears to be too weak to be detected by the in vitro pulldown assay, but is strong enough to localize endogenous E-cadherin at AJs. In contrast, p120 with either the K401M or N478A mutations resulted in complete loss of the p120-E-cadherin interaction

and significantly reduced expression of E-cadherin at the cell perimeter (Figure 3C) (Davis et al., 2003). The K401M or N478A mutants also failed to colocalize with endogenous E-cadherin when MDCK and MCF-7 cells expressing endogenous p120 were transfected with a vector expressing p120-1A-mRFP (Figure S3). Thus, our data strongly suggest that the newly revealed p120-JMD interface is crucial for colocalization of p120 with E-cadherin at AJs, and a minimal alteration within the JMD-binding site of p120 is sufficient to uncouple p120 from E-cadherin.

Uncoupling of the p120-N-Cadherin Interaction from p120-Rho-GTPase-Mediated Pathways

The dynamic regulation of cell-cell adhesion by p120 is not solely dependent on its interaction with the JMD, but also involves the association of p120 with numerous binding partners (Figure 1B). In neurons, synapse development and formation are highly dependent on the coordination of p120-dependent signaling processes, including N-cadherin retention, RhoA inhibition, and Fer-dependent dephosphorylation of β -catenin (Arikath and Reichardt, 2008; Elia et al., 2006; Lee et al., 2008).

Prior work using p120 deletion mutants, Δ ARM-R3 and Δ 622–628, has demonstrated that the maturation of the dendritic spine

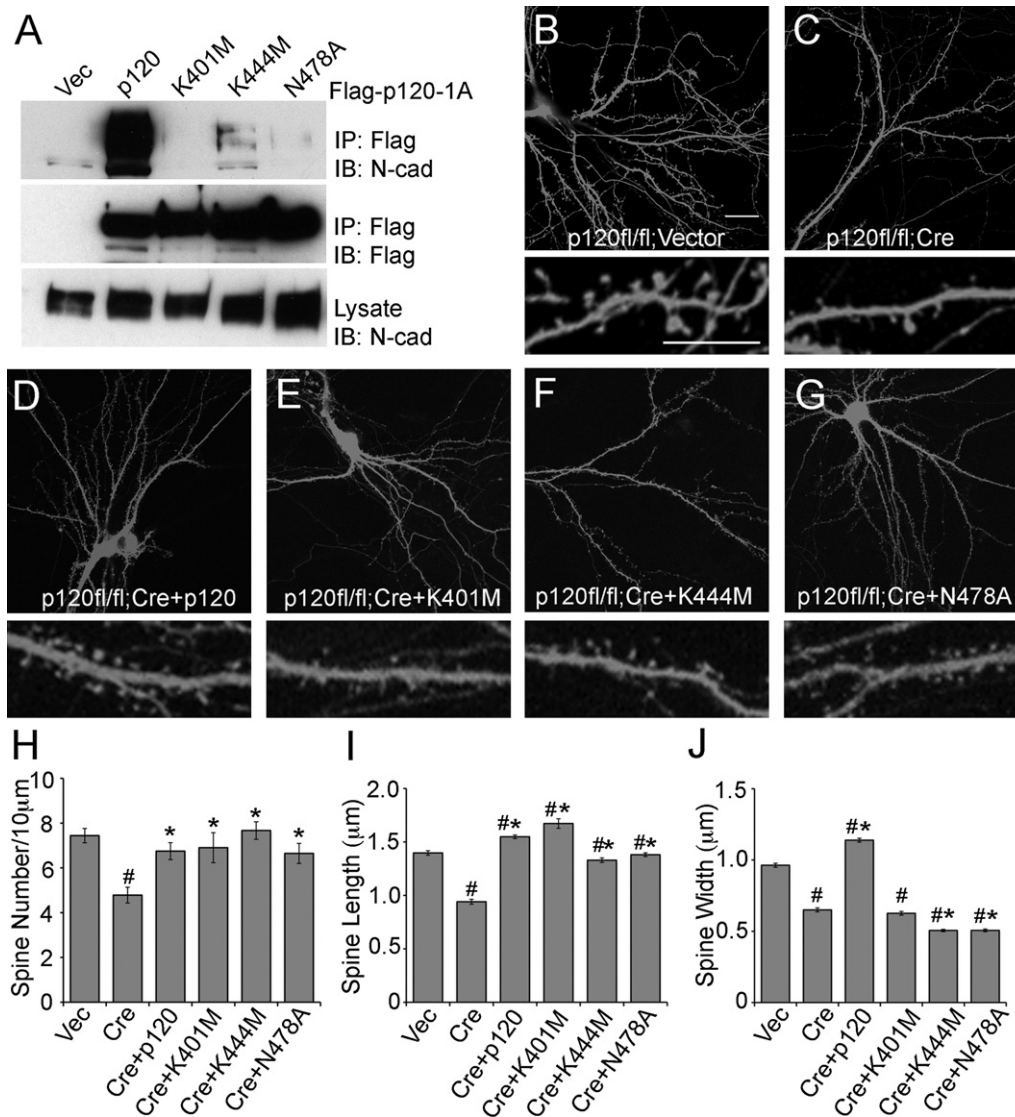


Figure 4. p120 Mutants Show Reduced Affinity for N-Cadherin, and Reduced Dendritic Spine Head Width in Neurons Lacking p120

(A) With transfected 293T cells, the interactions of N-cadherin with Flag-p120-1A (wild-type, K401M, K444M, or N478A) were examined in anti-Flag immunoprecipitates by anti-N-cadherin immunoblotting. As expression controls, N-cadherin levels were examined in total cell lysates.

(B–G) Hippocampal neurons from postnatal day 0 $p120^{fl/fl}$ ($p120^{fl/fl}$) mice were transfected at day 10 with the indicated constructs (Cre, p120 mutants) together with GFP and fixed at day 14. Representative images of transfected neurons are shown at low magnification (upper panels) and high magnification (lower panels).

(H–J) Densities of dendritic spines (H), mean spine length (I), and mean spine head width (J) were quantified. The description of dendritic spine is shown in Figure S4. Spines from three independent hippocampal cultures were measured. For (H), $n = 14$ (Vec), 15 (Cre), 18 (Cre+p120), 9 (Cre+K401M), 10 (Cre+K444M), and 12 (Cre+N478A). For (I) and (J), $n = 460$ (Vec), 358 (Cre), 635 (Cre+p120), 343 (Cre+K401M), 355 (Cre+K444M), and 413 (Cre+N478A). The scale bars represent 20 μm (low magnification) and 5 μm (high magnification). #, $p < 0.05$ versus Vec. *, $p < 0.05$ versus Cre. Error bars represent the standard error of the mean (SEM). See Table S2 for Student's *t* test results.

head width (Figure S4) requires a proper p120-N-cadherin interaction and the accumulation of spine density and growth of spine length require the p120 regulation of Rho-GTPases (Elia et al., 2006). However, creation of p120 $\Delta\text{ARM-R3}$ mutant involves removal of more than 40 residues from the ARM domain, which could have a detrimental effect on the structure and function of p120. Since we have confirmed that single-residue mutants of p120 (K401M, K444M, and N478A) are well folded and unable

to tightly associate with E-cadherin (Figure 3A), we decided to further test the role of p120 binding to cadherin in neuronal dendritic spine formation by expressing these mutants in hippocampal neurons cultured from $p120^{fl/fl}$ mice lacking p120. The inability of these p120 mutants to interact with N-cadherin was confirmed by coimmunoprecipitation of N-cadherin and p120 variants coexpressed in 293T cells (Figure 4A). When Cre recombinase expression was used to acutely knock down

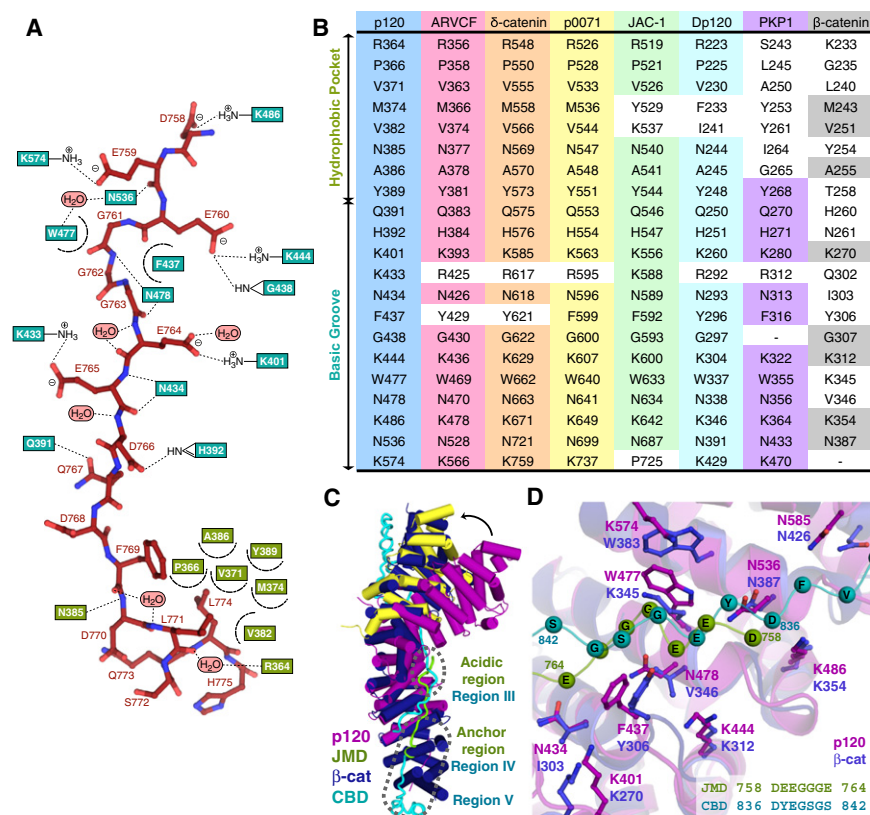


Figure 5. Conservation of the JMD_{core}-Binding Residues in p120, ARVCF, δ -Catenin, p0071, JAC-1, Dp120, PKP1, and β -Catenin

(A) Schematic representation of the interactions between the JMD_{core} and p120. p120 basic ARM groove residues are shown in blue boxes, and the N-terminal hydrophobic pocket residues are shown in green boxes. Ordered water molecules are shown as pink ovals. Hydrogen bonds are shown as straight dashed lines. Curved dashed lines indicate hydrophobic interactions.

(B) Conservation of the JMD_{core}-binding residues in p120, ARVCF, δ -catenin, p0071, JAC-1, Dp120, PKP1, and β -catenin. Colored boxes indicate residues identical to the JMD_{core}-binding residues in human p120.

(C) Superposition of the p120-4A_{ins}/JMD_{core} and β -catenin/CBD complexes (PDB code 117X). p120-4A_{ins} (purple) and β -catenin ARM domain (navy) are shown as cylinders. JMD (green) and CBD (cyan) are shown as tubes. p120 ARM R6–9 (yellow) can be independently superposed to β -catenin ARM R8–11 by rotating $\sim 30^\circ$.

(D) Comparison of the basic ARM grooves of p120 and β -catenin. The C _{α} atoms of the JMD_{core} (green) and CBD (cyan) are shown as spheres.

p120 expression at the stage of spine morphogenesis in cultured neurons from p120^{flx/flx} mice, the p120-deficient spines exhibited reductions in spine density, length, and head width compared to the control neurons (Figures 4B, 4C, 4H, 4I, and 4J). Expression of p120 WT, K401M, K444M, or N478A in p120-deficient neurons resulted in restoration of spine density and length (Figures 4B–4I). However, the reduced width of spine head observed in p120-deficient neurons was only restored by expression of p120 WT, not by the p120 mutants (Figures 4B–4G and 4J). These observations confirm the necessity of a p120-N-cadherin interaction during spine head maturation (Elia et al., 2006) but further illuminate the multifunctional roles of p120 by demonstrating that single-residue mutations in p120 (K401M, K444M, and N478A) can promote dendritic spine morphogenesis with nearly normal spine density and length, presumably via Rho-GTPase signaling, as previously described (Elia et al., 2006). In future experiments, it will be interesting to determine whether p120 and N-cadherin-dependent promotion of spine head maturation requires cadherin clustering mediated via the proposed p120/JMD-dependent mechanism (Figure 5I).

Distinct Cadherin-Binding Specificities of p120 and β -Catenin

Comparison of ARM domain sequences from p120 and other ARM domain-containing proteins revealed that the JMD-binding residues (Figure 5A) are highly conserved among p120 subfamily members in vertebrates (ARVCF, δ -catenin, and p0071), as well as invertebrate p120 proteins (Dp120 and JAC-1; Figure 5B). It also revealed unexpected conservation of the basic groove

residues in PKP1, which is not known to associate with the cadherin cytoplasmic tail through its ARM domain (Figure 5B). In contrast, only a few residues in the basic ARM grooves of p120 are conserved in β -catenin (β -catenin residues Lys270, Gly307, Lys312, Lys354, and Asn387; Figure 5B).

As p120 and β -catenin utilize their ARM domains to recognize the JMD and CBD, respectively, of E-cadherin cytoplasmic tail, a question arises as to how they distinguish two adjacently located binding sites (Figure 1D). When the p120-4A_{ins}/JMD_{core} and β -catenin/CBD complexes are superposed, p120 ARM R1–5 can be aligned with β -catenin ARM R3–7 with an RMSD of 1.52 Å over 150 C _{α} atoms (Figure 5C). Comparison of the p120/JMD_{core} and β -catenin/CBD complexes reveals that both catenins utilize a basic ARM groove and an exposed N-terminal hydrophobic surface to recognize charged and hydrophobic properties of the JMD and CBD, respectively (Figure 5C). However, these catenins utilize a distinct set of surface exposed residues to interact with the cadherin cytoplasmic tail, as only a few residues are conserved between p120 and β -catenin in the basic ARM grooves (Figures 5B and 5D). For example, the interface involving the triple glycine motif of the JMD and a trough within the p120 basic ARM groove does not exist between the CBD and β -catenin. In addition, the contiguous arrangement of electrostatic and hydrophobic interfaces is unique to p120, as the β -catenin-CBD interaction involves an additional phosphorylation-induced binding interface (region IV) that separates the electrostatic (region III) and hydrophobic (region V) interfaces (Figure 5C) (Huber and Weis, 2001). Thus, comparison of the distinct cadherin/catenin interfaces reveals that the

ligand-specific binding pockets, as well as the spatial arrangement of modular binding pockets within the ARM domains, determine distinct substrate specificities of p120 and β -catenin for E-cadherin.

Dynamic and Static Binding Interfaces between p120 and the JMD

Our structural and mutagenesis data explain the basis for the JMD_{core} mutations often used to uncouple p120 from E-cadherin (Thoreson et al., 2000). As an example, alanine substitution of the triple glycine motif (⁷⁶¹GGG⁷⁶³ → AAA) or the succeeding acidic residues (⁷⁶⁴EED⁷⁶⁶ → AAA) either indirectly or directly prevents the formation of salt bridges with lysines from p120 (Lys401 and Lys433; Figure 5A). However, we could not provide any insight on the involvement of JMD residues 776–781 in the p120 interaction suggested by the pulldown assay (Figure 3A), prompting us to investigate the significance of regions outside of the JMD_{core}. More specifically, we examined the involvement of the endocytic LL motif (residues 743–744), tyrosine phosphorylation sites (residues 755–756), and Arg751 (a HDGC mutation site) in the direct association with p120.

Isothermal titration calorimetry (ITC) determined that p120-4A_{ΔIns} shows affinity in the submicromolar range for both the JMD_{ext} peptide (residues 736–781; $K_d = 0.6 \mu\text{M}$; Figure 6A) and the peptide without the N-terminal 11 residues (JMD_{ΔN}; residues 747–781; $K_d = 0.9 \mu\text{M}$). In contrast, p120-4A_{ΔIns} shows significantly reduced affinity toward the JMD_{core} ($K_d = \sim 40 \mu\text{M}$), indicating that the residues flanking the N and C termini of the JMD_{core} (residues 747–757 and 776–781) are necessary for tight binding with p120. We therefore performed NMR titrations of ¹⁵N-labeled JMD_{ext} with unlabeled p120-4A_{ΔIns} to map the p120-binding site within the JMD (Figures 6B–6H). The narrow range of ¹H chemical shifts observed for the JMD_{ext} indicated that the peptide in solution is unstructured (Figure S5), which is consistent with the previously observed property of the cadherin cytoplasmic tail (Huber et al., 2001). On the basis of observed signal broadening and chemical shift differences between free and bound states ($\Delta\delta_{B-F}$) in the ¹H-¹⁵N HSQC spectra of JMD_{ext}, we determined that three sections within the JMD_{ext} showed distinct modes of interaction with p120-4A_{ΔIns} (Figure 6B): residues 736–740 (section I) are in the fast exchange regime (small $\Delta\delta_{B-F}$) reflecting the N-terminal disordered region participating in weak association; residues 741–746 (section II) are in the slow-to-intermediate exchange regime (medium $\Delta\delta_{B-F}$) reflecting this proline-rich segment (containing the endocytic LL motif) participating in medium-strength association; and residues 747–781 (section III) are in the slow exchange regime (large $\Delta\delta_{B-F}$) reflecting this large segment of JMD_{ext} (containing Arg751, the tyrosine phosphorylation sites, the core region and the C-terminal residues 776–781) participating in strong association. On the basis of these observations, we have defined the JMD residues 747–781 as a “static” p120-binding site mainly responsible for the specific interaction between p120 and E-cadherin, and the JMD residues 736–746 as a “dynamic” p120-binding site where the intermediate-to-weak interaction with p120 provides moderate masking of the LL motif from the endocytic machinery proteins (see below). The orientation of the JMD_{core} within the complex structure and an extensive basic

ARM groove formed by ARM R1–7 (Figures 1C and 6I) suggest that a basic patch formed by ARM R5–7 is likely to interact with the JMD residues preceding the core region (residues 736–757; Figure 6B). The proposed interaction between the p120 ARM R1–7 and the JMD_{ext} is consistent with the previous studies by Ireton et al. (2002), which showed that individual deletion of ARM repeats 1–6 (repeat number based on our structure) abolished binding of p120 to the cadherin JMD.

Dynamic p120-JMD Interaction Determines the Fate of Cadherin

The present study has revealed that there are two types of p120-JMD interfaces, dynamic and static in binding nature. We propose that p120 contributes to the regulation of cadherin-mediated cell-cell adhesion by protecting the endocytic LL motif through the “dynamic” p120-JMD interface, while its association with E-cadherin is stabilized through the “static” p120-JMD interface (Figure 7). Although the “dynamic” interface does not significantly contribute to the affinity between p120 and the JMD (Figure 6A), the propensity of the LL motif to be released from the “dynamic” interface of JMD-bound p120 provides opportunities for clathrin adaptor complexes, such as AP2, to competitively bind to the LL motif and initiate the endocytosis of cadherins (Figure 7). Interestingly, the affinity between p120-4A_{ΔIns} and the JMD_{ext} peptide is similar to that reported for AP2 and an LL motif containing peptide ($K_d = 0.85 \mu\text{M}$) (Kelly et al., 2008). This suggests that the stability of E-cadherin at AJs is dependent on an equilibrium of cadherin retention promoted by p120, and cadherin internalization induced by the endocytic machinery. Further destabilization of the “dynamic” p120-JMD interface due to phosphorylation of p120 (Bauer et al., 1998) or allosterically increased affinity between endocytic proteins and the LL motif (Kelly et al., 2008) could lead to reduced levels of E-cadherin at the cell surface. Alternatively, cadherin internalization could also be induced by destabilization of the “static” p120-JMD interface. Src-dependent phosphorylation of the E-cadherin JMD at Tyr755 and Tyr756 within the “static” interface could either hinder association of p120 with the JMD and/or promote association of Hakai with the JMD, consequently displacing p120 from the cadherin-catenin complex at the AJ (Figure 7) (Fujita et al., 2002). The HDGC-associated R749W mutation of human E-cadherin (Kaurah et al., 2007) would also be expected to interfere with the stable binding of p120 with the JMD as a result of a change within the “static” interface. Taken together, our structural data provide insights into the significance of the p120-regulated stability of cadherin-catenin complexes in AJ formation, in neuronal synapse development, and in increased risks of diffuse gastric cancer.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

Recombinant p120 proteins and the JMD peptides used in this study were individually expressed as GST-fusion proteins in *Escherichia coli* and purified with the glutathione-sepharose resin (GE Healthcare). Subsequently, GST was cleaved off by thrombin and the cleaved protein (p120-4A_{ΔIns} or JMD) was further purified by size-exclusion chromatography. The purified proteins were exchanged into protein storage buffer (50 mM Tris-HCl [pH 7.0], 150 mM NaCl, 1 mM TCEP-HCl). See the Extended Experimental Procedures for further details.

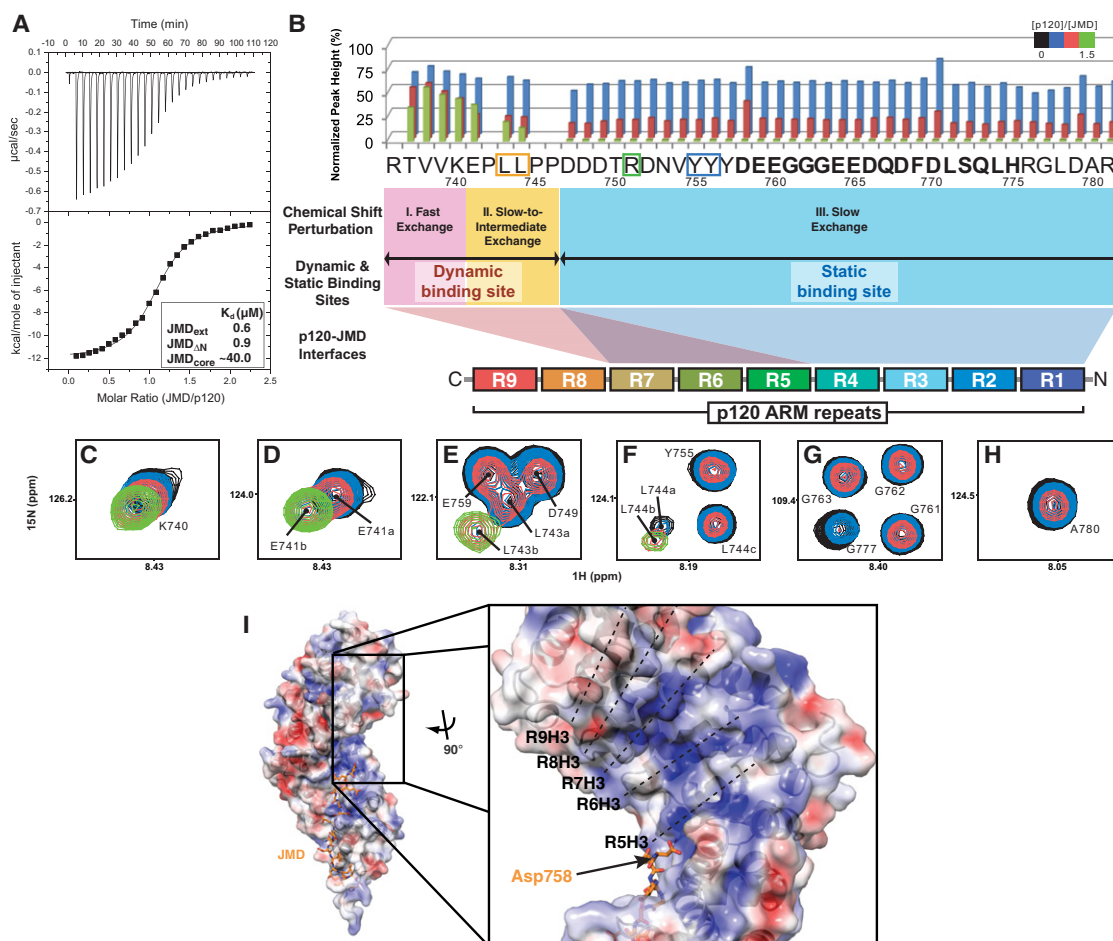


Figure 6. Dynamic and Static Binding of p120 with the JMD of E-Cadherin

(A) Calorimetric titration analysis of the p120-4A Δ Ins-JMD interaction. p120-4A Δ Ins binds to the JMD_{ext} with K_d of 0.6 μ M and the stoichiometry of 1.1 mole JMD/mole p120-4A Δ Ins. In comparison, p120-4A Δ Ins interacts with the JMD Δ N and the JMD_{core} with K_d of 0.9 μ M and \sim 40 μ M, respectively.

(B–H) NMR titration analysis of 15 N-labeled JMD_{ext} peptide binding to unlabeled p120-4A Δ Ins. 1 H- 15 N HSQC spectra of 15 N-JMD_{ext} were collected at titration steps corresponding to the [p120]/[JMD] molar ratio of 0 (100% peak height not shown in B, black in C–H), 0.5 (blue), 1 (red), and 1.5 (green). Complete backbone amide assignment of 15 N-JMD_{ext} is shown in Figure S5. Normalized peak heights of 15 N-JMD_{ext} from the HSQC spectra are plotted against the JMD_{ext} residues 736–781 (B). On the basis of the NMR behavior of JMD residues during titration, the JMD_{ext} was divided into three sections: I, II, and III (colored in pink, yellow, and blue, respectively). In section I, peaks corresponding to residues 736–740 were gradually shifted from the free state (black peak) to the p120-bound state (green peak), indicating a fast exchange regime on a NMR time scale (e.g., K740 in C). Section II consists of residues 741–746 and peaks corresponding to the free and p120-bound states of Glu741 (E741a and E741b in D), Leu743 (L743a and L743b in E), and Leu744 (L744a and L744b in F) were separately observed during titration, indicating a slow-to-intermediate exchange regime. In section III, peaks corresponding to the free state of residues 747–781 (e.g., D749 in E, E759 in E, Y755 in F, G761 in G, G762 in G, G763 in G, G777 in G, and A780 in H) experienced extensive signal broadening, while peaks corresponding to the p120-bound state were undetected by NMR (the p120-4A Δ Ins/JMD_{ext} complex is \sim 70 kDa), indicating a slow exchange regime. An additional peak for Leu744 also experienced extensive broadening during titration (L744c in F). On the basis of these observations, we defined that JMD residues 736–746 constitute the “dynamic” p120-binding site and JMD residues 747–781 constitute the “static” p120-binding site. The JMD_{core} residues are shown as bold letters. The yellow box indicates the endocytic LL motif. The green box indicates Arg751 (the R749W mutation site in human E-cadherin). The blue box indicates tyrosine phosphorylation sites.

(I) The basic ARM groove formed by the p120 ARM R5–7 involves R5H3, R6H3, and R7H3 helices (indicated by the dashed lines in the close-up view).

Crystallization and Data Collection

Crystals of the p120-4A Δ Ins/JMD_{core} complex were grown at 293 K by vapor diffusion. The protein solution, containing p120-4A Δ Ins (20 mg/ml) and four molar excess of the JMD_{core} peptide, was mixed with an equal volume of the reservoir solution containing 0.1 M Bis-Tris (pH 5.6), 1.8 M (NH₄)₂SO₄, and 3% methanol for form I crystals, or 0.1 M HEPES (pH 7.4) and 8% (w/v) PEG-8000 for form II crystals. Diffraction data were collected at the Advanced Photon Source beamline 19BM and processed with HKL2000 (Otwinowski and Minor, 1997). See the Extended Experimental Procedures

for further details. Statistics pertaining to the diffraction data are presented in Table S1.

Structure Determination and Refinement

The structure solution for the p120-4A Δ Ins/JMD_{core} complex structure was solved by molecular replacement with the PKP1 ARM domain used as a search model. Successive rounds of manual model building and refinement were performed to refine the models of p120-4A Δ Ins and the JMD_{core}. Refinement statistics are presented in Table S1. See the Extended Experimental

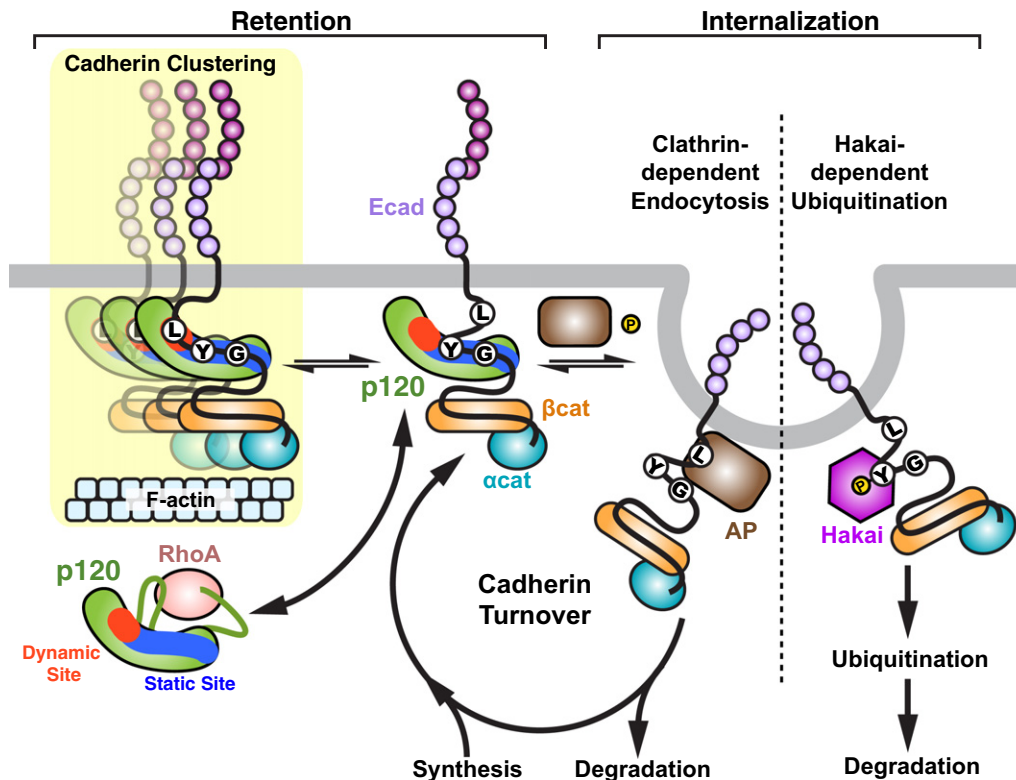


Figure 7. Scheme of p120-Dependent Regulation of the Stability of Cadherin-Catenin Complexes at the Cell Surface

The cadherin-catenin complex consists of E-cadherin (Ecad), p120, β -catenin (β cat), and α -catenin (α cat). p120 associates with the cadherin JMD, which contains the endocytic LL motif (L), tyrosine-phosphorylation sites (Y), and triple glycine motif (G), through the dynamic (red) and static (blue) binding sites. Adaptor proteins (AP) and Hakai recognize the exposed LL motif and phosphorylated (P) tyrosines, respectively, to facilitate endocytic processing of cadherin-catenin complexes. Cadherin clustering is likely to require a high local concentration of cadherin-catenin complexes engaged in *trans*-dimerization and involve synergistic interactions occurring on both sides of the plasma membrane: the JMD-induced p120 oligomerization occurring on the cytoplasmic side and the *cis*-interaction of *trans*-dimerized cadherin ectodomains occurring on the extracellular side.

Procedures for further details. Molecular graphics representations were prepared with PyMOL (DeLano Scientific).

GST-Pulldown Assays

GST-fusion protein (GST-JMD [residues 747–781], GST-JMD Δ C [residues 747–775], or GST) was bound to the glutathione-sepharose beads, mixed with purified p120-4A Δ ins (WT or mutant), and nonbound material was washed away from the beads. The bound protein mixture was eluted with a buffer containing 10 mM reduced glutathione, resolved by SDS-PAGE, and analyzed by Coomassie staining.

Circular Dichroism

Data were collected in 1 nm increments (20 nm/min) with 0.01 or 0.1 cm pathlength cuvette on a Jasco J-815 CD Spectrometer (Jasco) at 293 K.

Mammalian Cell Culture, Transfection, and Immunofluorescence

Stable p120-knockdown monoclonal cell lines of MDCK and MCF-7 cells were established through the use of a vector expressing short hairpin RNA (shRNA) directed against endogenous p120 and a puromycin resistance gene. For fluorescence microscopy, cells grown to 80% confluency were transfected with a modified pcDNA3.1 vector containing p120-1A-mRFP and further incubated for 24 hr. Immunostaining of the fixed, pretreated cells was performed by incubation with appropriate primary and secondary antibodies for 1 hr at 293 K. Anti-E-cadherin mouse monoclonal (BD Transduction Laboratories) and goat anti-mouse Alexa 488 (Invitrogen) were used as primary and

secondary antibodies. See the **Extended Experimental Procedures** for further details.

Immunoprecipitation, Immunoblotting, Primary Hippocampal Culture, Gene Transfer, Imaging, and Statistical Analysis

293T cells were transfected with vector or Flag-tagged p120 constructs together with N-cadherin using Fugene-6 (Roche Applied Science) and lysed 3 days after transfection. Lysates were clarified by centrifugation, incubated with anti-Flag antibody, and immunoprecipitated with Protein A Sepharose CL-4B (GE Healthcare). The immunoprecipitates were fractionated by SDS-PAGE and analyzed by immunoblotting.

Dissociated hippocampal neurons were prepared from postnatal day 0 (P0) p120^{fllox/fllox} mice as described previously (Elia et al., 2006), using procedures approved by the University of California, San Francisco, committee on Animal Research. Cells were transfected with a vector containing p120-1A at day 10, and fixed for confocal imaging at day 14. Nine to 15 neurons from three different cultures were examined per each condition. Student's *t* tests were performed to assess statistical significance (Table S2). See the **Extended Experimental Procedures** for further details.

Isothermal Titration Calorimetry

Calorimetric titrations were carried out on a Microcal VP-ITC titration calorimeter (MicroCal) at 293 K. A protein solution (14–50 μ M) was titrated with a peptide solution (160–500 μ M) in 26 steps of 10 μ l. Data were analyzed with a one-site binding model with the Origin software package supplied by MicroCal.

NMR Spectroscopy

Backbone assignment of the $^{15}\text{N}/^{13}\text{C}$ labeled JMD_{ext} was accomplished with a set of standard 3D triple-resonance experiments. ^1H - ^{15}N HSQC spectra were collected during titration of ^{15}N -JMD with unlabeled p120-4A $_{\Delta\text{Ins}}$. See the [Extended Experimental Procedures](#) for further details.

Sequence Alignment

Multiple sequence alignment was performed with T-Coffee (Notredame et al., 2000). See the [Extended Experimental Procedures](#) for further details.

ACCESSION NUMBERS

Atomic coordinates and structure factors for the structures reported in this work have been deposited in the Protein Data Bank under PDB codes 3L6X and 3L6Y.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and two tables and can be found with this article online at [doi: 10.1016/j.cell.2010.01.017](https://doi.org/10.1016/j.cell.2010.01.017).

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