Conformational epitopes at cadherin calcium-binding sites and p120-catenin phosphorylation regulate cell adhesion

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ABSTRACT We investigated changes in cadherin structure at the cell surface that regulate its adhesive activity. Colo 205 cells are nonadhesive cells with a full but inactive complement of E-cadherin–catenin complexes at the cell surface, but they can be triggered to adhere and form monolayers. We were able to distinguish the inactive and active states of E-cadherin at the cell surface by using a special set of monoclonal antibodies (mAbs). Another set of mAbs binds E-cadherin and strongly activates adhesion. In other epithelial cell types these activating mAbs inhibit growth factor–induced down-regulation of adhesion and epithelial morphogenesis, indicating that these phenomena are also controlled by E-cadherin activity at the cell surface. Both types of mAbs recognize conformational epitopes at different interfaces between extracellular cadherin repeat domains (ECs), especially near calcium-binding sites. Activation also induces p120-catenin dephosphorylation, as well as changes in the cadherin cytoplasmic domain. Moreover, phospho-site mutations indicate that dephosphorylation of specific Ser/Thr residues in the N-terminal domain of p120-catenin mediates adhesion activation. Thus physiological regulation of the adhesive state of E-cadherin involves physical and/or conformational changes in the EC interface regions of the ectodomain at the cell surface that are mediated by catenin-associated changes across the membrane.

INTRODUCTION

The dynamic regulation of cadherin-mediated adhesion is important for many morphogenetic and physiological processes, including cell rearrangements during embryonic development, junctional remodeling for epithelial morphogenesis, cell invasion during cancer metastasis, wound healing, and regulation of endothelial barrier function during inflammatory responses (Gumbiner, 2005; Dejana et al., 2008; Vestweber et al., 2010; Baum and Georgiou, 2011; Takeichi, 2011). Although transcriptional regulation often underlies cadherin loss or switching, there is abundant evidence that cadherins are regulated rapidly and posttranslationally during many of these processes. Several different mechanisms have been proposed to mediate dynamic regulation of cadherin adhesion, including internalization and trafficking via endocytosis (Lu et al., 2003; Ulrich et al., 2005; Ogata et al., 2007; Baum and Georgiou, 2011), tyrosine phosphorylation and dissociation of catenins, and cytoskeletal interactions (Brembeck et al., 2004; Lilien and Balsamo, 2005; Daugherty and Gottardi, 2007). There is also evidence that cadherin adhesion activity on the cell surface is directly subject to regulation, independent of internalization or catenin dissociation (Brieher and Gumbiner, 1994; Aono et al., 1999; Zhong et al., 1999; Chen et al., 2009).

Regulation of cell adhesion requires the breaking and remaking of homophilic adhesive bonds, and even regulation by internalization and trafficking requires control of cadherin disengagement from the homophilic bond between neighboring cells. Although much has been learned about the signaling pathways, catenins, and cytoskeletal proteins that affect adhesion, the mechanisms by which they regulate cadherin homophilic binding on the cell surface is unknown. This contrasts greatly with our understanding of the regulation of integrin-mediated adhesion, for which it is known that conformational changes in the integrin extracellular domain and associated changes...
in clustering and ligand binding affinity are controlled by associated cytoplasmic proteins such as talin and kindlins in response to signaling events (Hynes, 2002; Ginsberg et al., 2005; Shattil et al., 2010). This is probably due to the lack of experimental systems that lend themselves to such studies of changes in cadherin binding activity, in contrast to the integrins, which are rapidly activated during triggering of platelet aggregation or leukocyte adhesion.

A great deal of progress has been made understanding the high-resolution structure of the classical cadherin ectodomain and the structural basis of the cadherin homophilic bond. The x-ray crystal structure of the entire C-cadherin ectodomain provides a model for understanding cadherin structure and binding properties (Boggon et al., 2002). A critical Trp-2 residue in the N-terminal extracellular cadherin repeat domain 1 (EC1; or Trp-2 and Trp-4 for type II cadherins; Patel et al., 2006) mediates strand exchange between cadherins to form a dimer, and this key dimer interaction is believed to represent the adhesive homophilic bond between cadherins on neighboring cells. This bond is generally believed to represent a strong state of adhesion.

Several biophysical and structural findings suggest that additional interactions between cadherin ectodomains might occur. Surface force and atomic force measurements have provided evidence for three different interactions (Zhu et al., 2003; Shi et al., 2010). An electron microscopic study of a fragment of VE-cadherin suggests that it forms a stable hexamer (Hewat et al., 2007), although such structures have not been observed in other studies (Brasch et al., 2011). An alternative dimer (X-dimer) involving the calcium-binding interface between EC1 and EC2 domains has also been observed in x-ray crystal structures of cadherins, and this X-dimer may form as an intermediate in the formation of the final Trp-2–dependent adhesive bond (Harrison et al., 2010; Hong et al., 2011). However, little is known about how the structure of the cadherin ectodomain may change during the physiological regulation of adhesive states on cells.

We provided evidence that physiological regulation of C-cadherin in response to growth factors during embryonic morphogenesis involves changes in the adhesive state of cadherins at the cell surface, without changes in either expression levels at the cell surface or amounts of associated catenins (Brieger and Gumbiner, 1994; Chen and Gumbiner, 2006; Chen et al., 2009; Zhong et al., 1999). We hypothesized that the adhesive functions of cadherins, like those of the integrins, could be regulated by changes in conformation and/or physical state at the cell surface. To try to detect such changes, we raised mAbs to purified human E-cadherin ectodomain and screened for ones that could differentially recognize E-cadherin on the cells in the inactive or active adhesive states. The vast majority of mAbs out of more than 100 tested did not distinguish between the two states, exhibiting virtually identical binding curves in enzyme-linked immunosorbent assays (ELISA; Figure 1, left). This finding confirms that the amount of E-cadherin on the cell surface does not change during activation of adhesion and demonstrates that accessibility of the antibodies to E-cadherin is not limited during adhesion and subsequent cell compaction and epithelialization.

Five different mAbs did exhibit differential binding between untreated and activated Colo 205 cells when determined with a quantitative ELISA (Figure 1, right). In all five cases the binding was reduced in the activated state. Because general antibody accessibility is not limiting during activation, this finding suggests that certain E-cadherin epitopes selectively undergo some change in either conformation or local physical masking during adhesion activation. It is not clear why the reduction in binding is only partial; it could be due either to a large change in mAb binding to a small fraction of the total pool of E-cadherin molecules at the cell surface or to a small change in mAb binding to all or most of the E-cadherin molecules. We do not know how many E-cadherin molecules must be activated to induce changes in Colo 205 adhesion and compaction. Although E-cadherin did accumulate at cell–cell contact regions of activated Colo 205 cells, a large fraction did not redistribute out of the free surface at the edge of cell colonies (Figure 2A), suggesting that it may not all be activated or engaged in cell adhesion. Nonetheless, these and other findings (see Discussion) indicate that E-cadherin undergoes some sort of change in physical state and/or conformation during adhesion activation.

Direct activation of E-cadherin adhesion at the cell surface through mAb binding

We also sought mAbs that could bind to E-cadherin at the cell surface and induce a change in state and activate its adhesive function. To do so, we screened for mAbs raised to purified human E-cadherin ectodomain that could trigger adhesion and compaction of Colo 205 cells, similar to treatment with low levels of trypsin or staurosporine. Although the vast majority of anti-E-cadherin mAbs were either neutral or in fact inhibitory for adhesion (unpublished data; Table 1), we did obtain several mAbs that could trigger the formation of epithelial morphology (Figure 2A). Four such mAbs were strong activating mAbs—they effectively triggered the morphological change within 4 h; two others were considered weak activating mAbs, since they triggered a less extensive change (Table 1).
Monovalent antigen-binding fragment of antibody (Fab) prepared from the mAbs retained activating activity (Figure 2A), which rules out the possibility that they facilitate adhesion simply by cross-linking E-cadherin molecules between two neighboring cells. Thus these mAbs appear to induce a change in E-cadherin that leads to activation of adhesion.

To determine whether the activating mAbs act through specifically enhancing E-cadherin–mediated adhesion, we performed E-cadherin–specific adhesion assays. A flow adhesion assay was used in which strength of cell attachment to a substrate coated with purified E-cadherin protein is measured by the shear flow required to detach cells (Chappuis-Flament et al., 2001). Under control untreated conditions, Colo 205 cells failed to attach significantly to the E-cadherin substrate (Figure 2B). Treatment of Colo 205 cells with low levels of trypsin stimulated adhesion to the E-cadherin substrate, as evidenced by the increase shear flow required for cell detachment. Activating mAb 19A11 stimulated adhesion to the E-cadherin substrate even more, indicating that the mAbs work by directly and specifically triggering E-cadherin adhesive activity. Furthermore, activating mAbs were able to cause a change in cell surface E-cadherin that is detected by the distinguishing antibody (Figure 2C), suggesting that the activating mAbs trigger similar physical changes in E-cadherin to those due to staurosporine and low levels of trypsin.

Roles of cell surface E-cadherin activity in epithelial morphogenesis

Although dynamic regulation of cadherin adhesive function in response to morphogens is an important mediator of tissue morphogenesis (Meiners et al., 1998; Gumbiner, 2005; Ulrich et al., 2005), activation of E-cadherin from a completely inactive state probably occurs more rarely; compaction of the 8- to 16-cell-stage mouse embryo is the one obvious example (Vestweber et al., 1987; Fleming and Johnson, 1988). Therefore we wanted to determine whether the activity states controlled by our activating mAbs have roles in the more common phenomenon of partial adhesion down-regulation by growth factor signaling. Such down-regulation occurs during processes involving cell rearrangements, such as convergent–extension movements in gastrulating embryos (Zhong et al., 1999; Ulrich et al., 2005; Chen and Gumbiner, 2006; Ogata et al., 2007), wound closure and healing (Gorfinkiel and Arias, 2007), and junctional remodeling during epithelial morphogenesis/tubulogenesis (Meiners et al., 1998; Pollack et al., 1998; Baum and Georgiou, 2011).

We first tested whether activating mAbs had any effect on the regulation of adhesion by growth factors using A431 cells, an epidermal growth factor (EGF)–responsive human epithelial cell line. Using the flow adhesion assay, we observed that A431 cells exhibited reduced adhesion strength in response to EGF treatment (Figure 3A). Treatment of the cells with activating mAb along with EGF significantly strengthened adhesion. E-cadherin is known to inhibit cell motility and scattering in epithelial monolayers, and scattering can be enhanced by growth factors, in part through down-regulation of E-cadherin adhesion (Birchmeier, 1995; Meiners et al., 1998; Lu et al., 2003; Gumbiner, 2005). We therefore asked whether...
Epitope mapping reveals antibody recognition of interface regions between cadherin domains

To begin to determine the molecular and structural basis for the regulation of E-cadherin activity states, we mapped the epitopes recognized by distinguishing and activating mAbs. Initially a rough mapping to the cadherin EC domain was performed by immunoblotting using a set of expressed human E-cadherin deletion constructs (Supplemental Figure S1). The findings are summarized in Table 1. The EC domains recognized by the different independent mAbs tended to cluster, with four strongly activating mAbs recognizing EC1, two weakly activating mAbs recognizing EC3, and four distinguishing mAbs recognizing EC4 (one recognizes EC1-2). Moreover, binding of the distinguishing mAbs and the weak activating mAbs, but not that of the strong activating mAbs, depends on calcium (Table 1), suggesting that distinguishing and weak activating mAb epitopes are associated with conformations of the cadherin controlled by calcium-binding sites.

We then carried out a fine mapping of the epitope(s) recognized by the strongly activating mAbs. In vitro–translated deletion constructs of the EC1 domain mapped the C-terminus required for binding of the four mAbs to the very C-terminal end of EC1, to either T97 or V98 (Figure 5A). It was surprising that the four mAbs failed to bind a construct lacking the N-terminal residues of EC1 (unpublished data), suggesting that they recognize conformational epitopes comprising discontinuous segments. We therefore decided to examine residues over the entire length of EC1. Of
isotype
No
Weak activator, recognizes only native protein
EC3
IgG2a
Recognizes only native protein
EC4
IgG1
No
IgG1
No
EC1/2
Recognizes only native protein
Recognizes denatured ECad
ND
EC1
V98, R70, P16
IgG1
EC1
Recognizes denatured ECad
IgG1
EC3
Recognizes denatured ECad
Comment
V98, R70, E64
V323_A329
IgG1
IgG1
ND
IgG2a
S342, V434
IgG1
Recognizes denatured ECad
ND
ND
EC3
EC5
Weak activator, recognizes denatured ECad
V98, R70, E64
Recognizes only native protein
S432_V434
V98, R70, E64, P16
IgG2b
Recognizes denatured ECad
Recognizes denatured ECad
ND
EC1
S342, S432_V434
Full
Weak activator, recognizes only native protein
EC3
V323_A329
Partial

to the pre-pro and three N-terminal domains expressed in mammalian
cells. C-terminal deletions of the EC3 domain were examined for
mAb binding by using immunoblotting (Supplemental Figure S2). Both
weak activating mAbs required residues 329–332 at the boundary
between EC3 and EC4 for binding, which overlaps with the
calcium-binding site between EC3 and EC4 (Figure 6, B and B′),
consistent with partial or full dependence of the binding of these
two mAbs on calcium (Table 1). Although we have not yet deter-
mined whether other loops from EC3 that contribute to the calcium-
binding site are part of the epitope, these findings suggest that
similar to the strongly activating mAbs, these mAbs activate
E-cadherin adhesive function by binding to a calcium binding site,
albeit a distinct one.

Fine mapping of the epitope recognized by the four EC4-recog-
nizing distinguishing mAbs described in Figure 1 was done similarly
using mutations of secreted mammalian expression constructs con-
taining the pre-pro and four N-terminal domains (Figure 5A′). First,
the C-terminal region required for binding was mapped using a series of C-terminal deletion
constructs. All four distinguishing mAbs required residues 429–434 at the very C-termi-

| Antibody | Isotype | Epitope location | Amino acids involved in recognition | Calcium dependence | Comment | Comment |
|----------|---------|------------------|-----------------------------------|--------------------|---------|---------|
| 19A11    | IgG1    | EC1              | V98, R70, P16                     | No                 | Recognizes denatured ECad |
| 59D2     | IgG1    | EC1              | V98, R70, E64                     | No                 | Recognizes denatured ECad |
| 66E8     | IgG1    | EC1              | V98, R70, E64                     | No                 | Recognizes denatured ECad |
| 74A2     | IgG1    | EC1              | V98, R70, E64, P16               | No                 | Recognizes denatured ECad |
| 32H4     | IgG2a   | EC3              | V323_A329                        | Full               | Weak activator, recognizes denatured ECad |
| 70B4     | IgG3    | EC3              | V323_A329                        | Partial            | Weak activator, recognizes only native protein |
| 15A9     | IgG2a   | EC4              | S342, S432_V434                   | Partial            | Recognizes denatured ECad |
| 28E8     | IgG1    | EC4              | S342, S432_V434                   | Partial            | Recognizes denatured ECad |
| 29E2     | IgG1    | EC4              | S342, V434                       | Full               | Recognizes denatured ECad |
| 34G4     | IgG1    | EC1/2            | ND                                | Partial            | Recognizes only secreted Fc-ECad in native gel |
| 74D1     | IgG1    | EC4              | S432_V434                        | Full               | Recognizes denatured ECad |
| 27D2     | IgG2b   | EC3              | ND                                | Full               | Recognizes denatured ECad |
| 37F1     | IgG1    | EC5              | ND                                | ND                 | Recognizes only native protein |
| 46H1     | IgG2a   | EC3              | ND                                | No                 | Recognizes denatured ECad |
| 76D5     | IgG1    | EC4              | ND                                | Full               | Recognizes denatured ECad |
| 52F9     | IgG1    | EC5              | ND                                | ND                 | Recognizes only native protein |
| 67G8     | IgG1    | EC5              | ND                                | ND                 | Recognizes only native protein |

mAbs used in this study or identified in the screen were characterized by binding to human E-cadherin (ECad) using Western blotting or ELISA. ND, not determined.

TABLE 1: Antibody summary.

importance, none of the activating mAbs required the Trp2 residue
involved in homophilic bond formation, since they all strongly rec-
ognize the W2A mutant protein (Figure 5A′). None of the mAbs
recognize mouse E-cadherin (not shown), presumably because
they were generated in mouse and the protein is strongly ex-
pressed in many tissues. Because human and mouse E-cadherin
are highly conserved (Figure 5A′), we were able to map the
epitopes recognized by independently mutating the small number
of nonconserved residues in EC1 to the mouse versions. This re-
vealed that the mAbs do recognize a discontinuous epitope, which
includes residues P16, E64, and R70 (Figure 5A) in addition to
T97/V98. Of note, these residues all lie near residues that contri-
ute to the calcium-binding site between EC1 and EC2 (Figure 5A′).

Mapping these residues onto the published x-ray crystal structure
of E-cadherin EC1-EC2 reveals that the epitope comprises three
different loops in EC1 that come together to form the EC1 side of
the calcium-binding site at the opposite end of the EC1 domain
from the Trp2 homophilic adhesion binding site (Figure 6A, A′). Of
interest, binding of this group of mAbs does not require calcium
(Table 1), indicating that the structural arrangement of these loops
in EC1 must be at least partially independent of calcium binding.
That all four strongly activating mAbs recognize very similar con-
formational epitopes implicates this structure of the E-cadherin
molecule in the control of adhesion activity.

The epitope in EC3 recognized by the weak activating mAbs was
also partially refined by using secreted protein constructs containing
the pre-pro and three N-terminal domains expressed in mammalian

mAb binding by using immunoblotting (Supplemental Figure S2).
this calcium-binding site, S342P, greatly reduces binding of a different subset of three of the four distinguishing mAbs (Figure 5, B′ and B″). Mapping these residues onto the published x-ray crystal structure of mouse E-cadherin reveals that the epitope comprises different loops from discontinuous parts of EC4 that form the EC4 side of the calcium-binding site (Figure 6, C and C). The binding of this group of mAbs does depend either partially or fully on the presence of calcium (Table 1). Therefore the distinguishing mAbs appear to recognize a conformational epitope of EC4 near the calcium-binding site between EC4 and EC5.

An activating mAb to Xenopus C-cadherin, AA5, reverses the down-regulation of C-cadherin adhesive function in response to morphogens and blocks morphogenesis of embryonic tissues (Zhong et al., 1999). The AA5 epitope had only been mapped to the EC5 domain of C-cadherin (Zhong et al., 1999), and we therefore decided to refine it. C-terminal deletion mutagenesis of the in vitro–translated C-cadherin EC5 domain shows that AA5 binding requires residues 533–540 at the very C-terminus of EC5 (Figure 7A). Moreover, the epitope appears to be conformational, because binding is completely abolished by reducing and alkylating the two intramolecular disulfide bonds near the C-terminus of this domain (Figure 7B), suggesting that it may comprise loops from discontinuous segments. Site-directed mutation of cysteine residues involved in the formation of these disulfide bonds greatly reduced AA5 mAb binding (Figure 7C). These disulfide bonds are unique to the EC5 domain and are conserved features of classic cadherins (Boggon et al., 2002). Although there is no calcium-binding site at the C-terminus of the EC5 domain, the epitope recognized by the AA5-activating mAb is located in an analogous position at the base of the domain (Figure 6, D and D′, diagram). Thus the C-cadherin–activating mAb may also recognize a potential interface region between domains, in this case the interface between EC5 and the transmembrane domain, although the structure of the latter domain is not known.

E-cadherin activation triggers biochemical changes across the plasma membrane in the catenins and the cadherin cytoplasmic tail

Previous work with Colo 205 activation by treatment with low-level trypsin or staurosporine showed that activation was associated with a decrease in the apparent molecular weight of p120-catenin on SDS–PAGE and implicated p120 in activation (Aono et al., 1999). Activating mAb treatment caused a shift in p120-catenin very similar to the one caused by low trypsin treatment (Figure 8A). The effect was specific to mAb activation, because neutral and blocking mAbs that bind well to E-cadherin did not cause the shift. The shifted p120-catenin band was enriched in digitonin lysates of the Colo 205 cells (Figure 8A) but was detectable in lysates made with a variety of other detergents (Figure 8B). A shift to a lower apparent molecular weight is consistent with dephosphorylation of p120-catenin during other detergents (Figure 8B). A shift to a lower apparent molecular weight is consistent with dephosphorylation of p120-catenin during activation. Indeed, using phospho-specific mAbs to p120-catenin (Xia et al., 2004), we find that activation is associated with dephosphorylation of S268, S288, and T310 (Figure 8C). Thus, activating mAbs specifically affect p120 dephosphorylation via direct binding to the cadherin ectodomain and therefore act like agonists for this adhesion-associated event.

The observed dephosphorylation of p120-catenin resulting from mAb-induced activation could either be a direct effect of mAb treatment that may play a role in adhesion activation or an indirect consequence of cell adhesion resulting from activation. The time course of the mAb-induced shift in p120-catenin was similar to the time course of the development of strong adhesions detected by morphological observation, beginning in 1–2 h and fairly complete by 5 h (Figure 8D); therefore it is not possible to discern which happens first. However, we were able to separate activation from the development of overt adhesion by using a blocking mAb to E-cadherin. HECD1 completely blocked the ability of activating mAb to trigger adhesion (Supplemental Figure S3) but did not prevent the
molecular weight shift in p120-catenin (Figure 8E). Therefore the activating mAb is able to trigger activation-associated biochemical changes across the membrane independent of the formation of the adhesive bond between cells. This suggests that activating mAb binding induces a transmembrane change in the E-cadherin cytoplasmic domain that leads to the dephosphorylation of p120-catenin, which in turn may be involved in eliciting changes in E-cadherin to activate adhesion.

Indeed a transmembrane change in E-cadherin caused by activating mAb is able to trigger activation-associated biochemical changes across the membrane independent of the formation of the adhesive bond between cells. This suggests that activating mAb binding induces a transmembrane change in the E-cadherin cytoplasmic domain that leads to the dephosphorylation of p120-catenin, which in turn may be involved in eliciting changes in E-cadherin to activate adhesion.

FIGURE 4: Activating mAb enhances adhesion in MDCK cells and inhibits growth factor–induced epithelial tubulogenesis. (A) Tubulogenesis assay. MDCKII cells were cultured in type I collagen gels containing HGF and treated with neutral mAb, activating mAb, or no mAb. Cells were grown for 7 d, and phase contrast pictures were taken to assess morphology. Immunofluorescence staining for E-cadherin shows epithelial organization. (B) Activating mAb increased E-cadherin–mediated adhesion in MDCKII cells treated with HGF. Cells were either untreated or treated overnight with 3 µg/ml 19A11 activating mAb in a medium containing 20 ng/ml HGF. Cell adhesion strength was evaluated using increasing laminar flow to determine the force required to detach cells. (C) Activating mAb decreased rate of scratch wound closure in MDCKII monolayers. Wounded cultures were treated with 3 µg/ml neutral or activating mAbs, and wounds were allowed to close in medium with or without HGF for 14 or 24 h, respectively. Error bars, SD of two independent experiments (N = 8). Significance of difference was determined by Student’s t test; ns, no significant difference with p > 0.05; **p < 0.01; ***p < 0.005.

ectodomain mAbs (the low levels of cytoplasmic staining may be background). Activation of adhesion, by activating mAb, staurosporine, or low trypsin, stimulated the appearance of staining by the BD cytoplasmic domain mAb at the plasma membrane, as revealed by colocalization with anti-ectodomain immunostaining. Epitope mapping of the region recognized by the BD clone 36 mAb (Supplemental Figure S4) indicates that the epitope is at or near portions of the p120-catenin–binding region, as determined previously by mutagenesis experiments (Thoreson et al., 2000), as well as the crystal structure of the p120-cadherin complex (Ishiyama et al., 2010). Thus adhesion-activating mAbs trigger the unmasking of an epitope at the p120-catenin–binding region of the E-cadherin cytoplasmic domain.

Previous work with Colo 205 activation by treatment with low trypsin or staurosporine implicated p120-catenin functionally in adhesion activation (Aono et al., 1999). Because our activating mAb induced similar changes in p120-catenin phosphorylation, as well as changes in or near the p120-binding domain of E-cadherin, we asked whether mAb activation depends on p120-catenin interactions with the cadherin cytoplasmic domain. To do so, we used E-cadherin–deficient cells expressing a variety of E-cadherin mutant constructs (Figure 10). E-cadherin–deficient MDA-MB-231 cells were previously transfected to stably express wild-type E-cadherin and several different cytoplasmic domain mutant constructs (Wong and Gumbiner, 2003; Kim et al., 2011). The parental MDA-MB-231 cells do not adhere to purified E-cadherin in the flow adhesion assay (Figure 10, top, curve with triangles), nor do they exhibit morphologically discernible cell–cell adhesion in culture (unpublished data). Activating mAb increases the adhesion of cells expressing full-length E-cadherin to E-cadherin, as evidenced by increased resistance to detachment by shear flow (Figure 10, top). The activating mAb also increased the adhesion of cells expressing a construct lacking the β-catenin–binding domain and one with α-catenin sequences directly fused to the cytoplasmic domain. These results were observed for the cadherin construct expressing MDA-MB-231 cells using the wound closure assay (Supplemental Figure S5). As in Figure 3, activating mAb slows the scratch wound closure by cells expressing full length E-cadherin. The activating mAb also slowed the rate of wound closure by cells expressing a construct lacking the β-catenin–binding domain and one with α-catenin sequences directly fused to the
Regulation of adhesion activation by p120-catenin phosphorylation

The findings in Figures 8–10 suggest that p120-catenin and, in particular, p120-catenin dephosphorylation are involved in the regulation of E-cadherin activation. We therefore tested whether dephosphorylated p120-catenin can activate E-cadherin and whether blocking p120-catenin dephosphorylation inhibits mAb induced E-cadherin activation. To do so, we expressed specific mutant forms of p120-catenin harboring mutations in the Ser/Thr sites that have been shown to be phosphorylated (Xia et al., 2003) and for which we observe dephosphorylation during activation (Figure 8). Either proteins harboring mutations in several Ser/Thr...
sites (Figures 11 and 12) or those with single-site mutations (Supplemental Figures S6 and S7) were analyzed. The effects of the multiple-site mutants were more penetrant. Mutant mouse p120-catenin proteins were expressed in Colo 205 cells using retroviral vectors (Figure 11) either in the presence of endogenous human p120-catenin (control small interfering RNA [siRNA]) or when endogenous human p120-catenin was substantially knocked down using siRNA; expressed mouse p120-catenin was detected with an antibody specific for the mouse version. The siRNA treatment effectively depleted endogenous human p120-catenin (Figure 11), and similar to observations in the literature, the levels of E-cadherin were significantly reduced due to the role of p120-catenin in stabilizing cadherin expression (Ireton et al., 2002; Davis et al., 2003; Xiao et al., 2005). Of importance, expression of mouse p120-catenin completely rescued the levels of E-cadherin expression, irrespective of whether it contained any of the Ser/Thr mutations, either the S → A phosphorylation-deficient mutants or the S → E phosphomimetic mutations (Figure 11). Thus the state of Ser/Thr phosphorylation had no detectable role in the well-known function of p120-catenin in the regulation of E-cadherin expression, similar to the results reported by Reynold’s group (Xia et al., 2006).

We then tested whether the Ser/Thr mutations had any effects on activation of E-cadherin–mediated adhesion in Colo 205 cells (Figure 12). Images are shown in part I of Figure 12 (A–H and A′–H′), and quantitation of this and two additional similar experiments is shown in part II of Figure 12 (with corresponding a–h and a′–h′). It is not surprising that siRNA depletion of p120-catenin blocked the activation of adhesion by activating mAb, as well as reducing the levels of both p120-catenin and E-cadherin (Figure 12, A′ and B′) compared with control cells, in which activating mAb caused cells to aggregate and compact compared with neutral mAb-treated cells (Figure 12, A and B). Cells expressing wild-type mouse p120-catenin behaved the same as normal cells, with activating mAb inducing aggregation and compaction compared with neutral mAb treatment (Figure 12, C, D, C′, and D′). It is striking that expression of a phosphorylation-defective mutant of p120-catenin with six Ser/Thr residues mutated to Ala (6S,T→A) caused constitutive aggregation and compaction of the cells, even without treatment with activating mAb (Figure 12, E and E′); activating mAb had no additional effect (Figure 12, F and F′). Constitutive activation occurred even without depletion of endogenous human p120-catenin (Figure 12E), suggesting that the effect of the mutant is dominant. The constitutive activation of adhesion by the 6S,T→A mutant is also evident after quantitation of the data to measure the number of cells present in different-size aggregates (Figure 12, part II). Wild-type p120-catenin–expressing cells aggregated in response to treatment with activating mAb (Figure 12, c, d, c′, and d′), but 6S,T→A–expressing cells aggregated constitutively (Figure 12, e, f, e′, and f′). These data suggest that the dephosphorylated forms of p120-catenin induced during E-cadherin activation (Figure 8) are sufficient to mediate adhesion activation.

To determine whether dephosphorylation is required for mAb-induced activation of adhesion, we similarly expressed phosphomimetic forms of mouse p120-catenin harboring S,T→E mutations. Our findings again indicate that the combination of four N-terminal S/T mutations (Figure 12) was more penetrant than individual-site mutations (Supplemental Figure S7). Of importance, unlike the S,T→A mutations, the S,T→E mutations did not constitutively activate adhesion, showing that they did not act as phosphorylation-deficient mutations but instead are likely to be phosphomimetics. Expression of 4S,T→E p120-catenin in the absence of endogenous human p120-catenin significantly inhibited the ability of the activating mAb to induce cell aggregation and compaction (Figure 12, G′ and H′). In the presence of endogenous human p120-catenin it partially blocked aggregation and compaction (Figure 12, G and H). These findings are confirmed by quantitation of the data, which indicates that the 4S,T→E mutants significantly blocked induction of **FIGURE 6**: Structural modeling of activity state-associated epitopes located at the interfaces between domains. (A, A′) Epitope for strongly activating mAbs located between EC1 and EC2, close to the calcium-binding site. (B, B′) Epitope for weakly activating mAbs located at the calcium-binding site between EC3 and EC4. (C, C′) Epitope for distinguishing mAbs located at the calcium-binding site between EC4 and EC5. (D, D′) Epitope for C-cadherin–activating mAbs AA5 located at the base of EC5 near the transmembrane domain. Amino acid residues involved in epitopes are shown in blue, calcium ions are shown in orange, and Trp-2 structure is shown in cyan.
cell aggregation by activating mAb treatment (Figure 12, part II, g, h, g', and h'), especially when endogenous human p120-catenin is depleted. Therefore expression of phosphomimetic mutants of p120-catenin shows that p120-catenin dephosphorylation is required for mAb-induced activation of adhesion.

We also examined whether phosphorylation of individual Ser/Thr sites in the N-terminus have especially important roles in adhesion activation of Colo 205 cells, and the results are summarized in Table 2. Both phosphorylation-deficient S→A (Supplemental Figure S6) and phosphomimetic S→E mutants (Supplemental Figure S7) of the best candidate sites, as determined by their dephosphorylation during activation (Figure 8) or by their presence in the multisite mutants (Figure 12), were examined using the same approach as for the multisite mutants.

The effects of the single-site mutations were weaker than those of the multisite mutations, but several sites showed statistically significant partial effects that could be discerned through the quantitative measure of cell aggregation. In particular, S268, S288, and to a lesser extent S312 were found to have roles in constitutive activation of adhesion (Supplemental Figure S6, c, c', e, e', and g) or in the inhibition of antibody-induced adhesion (Supplemental Figure 7, d', f', and h') compared with other Ser-site mutations. The fact that the multisite mutants had stronger effects in the assays suggests that phosphorylation of these sites works in combination to regulate E-cadherin–mediated adhesion.

To determine whether phosphorylation of these sites has a more general role in regulating E-cadherin–mediated adhesion, we expressed the multisite 6S,T→A and 4S,T→E p120-catenin mutations in A431 cells in the presence of endogenous human p120-catenin (Figure 13). Both mutant proteins were expressed at similar levels as wild-type mouse p120-catenin (Supplemental Figure S8). As expected, expression of wild-type p120-catenin had no effect on the strength of adhesion to purified E-cadherin protein as assessed with the flow adhesion assay compared with mock-infected controls (Figure 13). Of interest, the 6S,T→A mutant significantly increased the strength of E-cadherin–mediated adhesion, whereas the 4S,T→E mutant significantly decreased the strength of adhesion (Figure 13). These findings suggest that the phosphorylation status of these Ser/Thr residues of p120-catenin have a general role in regulating the strength of E-cadherin adhesion, with phosphorylation decreasing overall adhesive strength.

**DISCUSSION**

Previous work on both Xenopus C-cadherin (Brieher and Gumbiner, 1994; Zhong et al., 1999; Chen et al., 2009) and human E-cadherin (Aono et al., 1999) provided evidence for the regulation of cadherin adhesion activity independent of cell surface expression levels. This led us in the present study to test the hypothesis that conformational changes or other physical changes (oligomerization/clustering) in the cadherin homophilic binding ectodomain are involved in the regulation of adhesion. We obtained evidence to support this hypothesis by two criteria—mAbs that distinguish activity states of E-cadherin, and mAbs that bind to E-cadherin and induce its activation at the cell surface.

The distinguishing mAbs identified in this study exhibited relatively small changes in binding to cell surface E-cadherin as a result of activation by staurosporine or activating mAbs. Nonetheless, the change in binding was very selective and observed only for a rare group of mAbs and therefore not simply due to changes in accessibility to the cell surface in general. Of importance, most of these mAbs recognized a very similar epitope, indicating that they target a very specific feature of the protein.

Adhesion-activating mAbs could work in either of two general ways, as exemplified by antibody regulation of integrin adhesive function (Schwartz et al., 1995; Stuiver and O'Toole, 1995; Humphries, 1996). One simple mechanism observed for integrin-activating mAbs is the stabilization of the high-affinity binding conformation for ligands, such as von Willebrand's factor. The other mechanism observed for integrins is more complex, with activating mAbs acting like signaling ligands that trigger changes in cytoplasmic factors that regulate integrin affinity state. The stimulation of p120-catenin dephosphorylation and the unmasking of the epitope near the p120-binding site suggest that cytoplasmic changes are similarly involved in antibody-induced E-cadherin activation. Of importance, these changes appear to be direct consequences of mAb binding rather than secondary effects subsequent to cadherin-mediated adhesion. Indeed, our functional analyses using mutations in the E-cadherin cytoplasmic domain and in
p120-catenin phosphorylation sites provide evidence that p120-catenin function is required for the activating mAb to enhance adhesion, consistent with studies using other activators (Aono et al., 1999). In this way, the activating mAbs can be considered to be agonists that trigger proximal events in the process of cadherin activation.

Our findings suggest that the regulation of cadherin adhesion activity involves the transduction of molecular changes throughout the entire cadherin molecule, entailing changes in homophilic binding via EC1, interfaces between several of the EC domains, and cytoplasmic signaling events via the catenins. We previously observed that the binding to a cadherin substrate causes cadherin clustering at the cell surface (Yap et al., 1998), which in turn seems to regulate the state of adhesion (Yap et al., 1997). Thus cadherins may undergo multiple physical or conformational changes back and forth across the plasma membrane during the overall process of cell adhesion, similar to those well documented for the EGF receptor (Macdonald-Obermann and Pike, 2009; Yang et al., 2010), and the activating mAbs may be able to induce some of these changes.

The epitopes recognized by the distinguishing and activating mAbs all map to interfacial regions between adjacent cadherin EC domains, especially at or near the calcium-binding regions between domains. The C-cadherin–activating mAb recognizes a region at a potential interface between the membrane-proximal EC5 domain and the transmembrane domain, even though this region does not contain a calcium-binding site. Moreover, most of the mAbs seem to recognize conformational epitopes, raising the possibility that conformational changes at interface regions and/or calcium-binding sites may be involved in the regulation of the adhesive-binding state. How potential changes at several different EC interfaces recognized by all these mAbs (EC1–EC2, EC3–EC4, EC4–EC5, EC5–transmembrane domain) regulate the state of the homophilic adhesive bond is not yet clear. One attractive hypothesis is that changes transmitted across multiple EC domain interfaces propagate an overall conformational change through the entire ectodomain to affect the distal homophilic binding site in EC1 and across the membrane to affect the cytoplasmic domain. There is evidence that changes in one EC domain can influence the properties of distal EC domains (Tsuiji et al., 2007; Shi et al., 2010). Similarly, we find that cytoplasmic processes are involved in mAb activation. It is not clear how cytoplasmic factors in the Colo 205 cells could control the state of Trp-2-mediated homophilic binding, preventing its formation in the absence of activation, without invoking some more complicated mechanism involving the whole ectodomain.

Our findings also provide direct evidence that phosphorylation of p120-catenin at specific Ser/Thr residues regulates adhesion activation in Colo 205 cells. Several specific N-terminal sites are dephosphorylated during mAb-induced adhesion activation. This appears to be directly associated with the transmembrane activation process, since dephosphorylation did not depend on the ultimate formation of adhesive contacts per se. Furthermore, the phosphorylation status of these same residues was found to control the state of activation. Blocking phosphorylation of these specific Ser/Thr residues in the N-terminus by mutations caused constitutive activation of adhesion, even in the presence of endogenous p120-catenin. This finding is similar to that observed by Aono et al. (1999) using a N-terminally truncated p120-catenin mutant, and it is possible that their results were due to the loss of these phosphorylation sites. Indeed we find that dephosphorylation of these N-terminal Ser/Thr sites may be involved in the regulation of the adhesive-binding state. How the phosphorylation status of these N-terminal Ser/Thr residues of p120-catenin can so strikingly regulate the activity state of E-cadherin at the cell surface is not yet clear. Although we observe an
Our findings have important implications for understanding the roles of cadherins in tissue morphogenesis and disease. For example, in evaluating the roles of cadherins in cancer it may be important to assess the activity state of the protein on the cell surface in addition to its level of expression. Furthermore, the ability to stimulate or maintain high cadherin activity at the cell surface may provide a novel way to enhance cadherin function to affect disease processes.

**MATERIALS AND METHODS**

**Cell culture and antibodies**

All cell lines used were from the American Type Culture Collection (Manassas, VA). Cells were cultured in standard CO₂/temperature conditions in medium containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA). EGF and HGF were purchased from PeproTech (Rocky Hill, NJ). E-Cadherin cytoplasmic tail-specific mAb (clone 36; 610182) was from BD Biosciences (San Diego, CA). The p120 C-terminus-specific rabbit mAb (2806-1) was from Epitomics (Burlingame, CA), and p120 phosphospecific mAbs (Xia et al., 2004) were kind gifts from Albert Reynolds (Vanderbilt University, Nashville, TN).

### Screening of hybridomas for activating and distinguishing mAbs

Mice were immunized with purified ECad1-5 protein described later, and hybridoma fusions were plated at low density in eighty 96-well plates to ensure that distinguishing mAb activity was not covered up by contaminating hybridomas secreting nondistincting mAbs. Supernatants of hybridomas were first screened for binding to the ectodomain of human E-cadherin using an ELISA described later. E-Cadherin-positive hybridoma supernatants were tested for binding to live Colo 205 cells with an ELISA for differential binding to nonactivated versus staurosporine-activated cells. For activating mAbs, hybridoma supernatants were screened for their ability to trigger adhesion and compact morphology, similar to what is observed as a result of activation with low-level trypsin or staurosporine (Aono et al., 1999). Clones of interest were checked for specificity using ELISA on pure E-cadherin protein, ELISA on live Chinese hamster ovary cells stably expressing either human E- or Xenopus C-cadherin, in a competitive cell ELISA with pure E-cadherin 1-5EC domain, by immunofluorescence staining, and by Western blot analysis.

### E-cadherin protein, expression, and epitope mapping

The E-cadherin ectodomain fused with the Fc region of human immunoglobulin G1 (IgG1) at the C-terminus (Fc-ECad1-5) was purified as described before (Chappuis-Flament et al., 2001). The Fc fragment was cleaved off by Factor Xa through an enzyme-specific site introduced into the fusion protein between ECad1-5 and Fc fragment. The latter was removed by protein A affinity chromatography. Mutations in the E-cadherin ectodomain were introduced using a QuikChange Site-Directed Mutagenesis kit associated change in the accessibility of a site in the cadherin cytoplasmic tail at or near the p120-catenin-binding site, activation is not associated with changes in the amount of p120-catenin or other catenins associated with the cadherin cytoplasmic domain (Aono et al., 1999; our present findings). Presumably, more subtle alterations in the structure of the cadherin cytoplasmic domain are involved.

Although the Colo 205 cell line used to identify the activating mAbs is atypical in being nonadhesive in the untreated state, the activating mAbs also affect growth factor-regulated adhesion in A431 and MDCK cells. In addition, their inhibition of cell migration, wound closure, and tubulogenesis suggests that regulation of cadherin activity state at the cell surface is important for tissue morphogenesis, similar to previously observed inhibition of the elongation of embryonic tissue via convergent extension by C-cadherin—activating mAbs (Zhong et al., 1999). Moreover, we observe a similar role for p120-catenin N-terminal Ser/Thr phosphorylation in the control of adhesive strength in the constitutively adhesive A431 cell line. Thus regulation of adhesion at the cell surface via physical changes in the cadherin protein may be a common feature of adhesive function in general.
germ in vitro system or into the pEE14 vector with a C-terminal Fc tag for transient transfection in Chinese hamster ovary cells. In the case of Chinese hamster ovary expression, the expressed proteins were purified from the cell culture media using protein A agarose.

Colo 205 cell activation assay
For the activation assay, Colo 205 cells were seeded on 96-well plates precoated with rat-tail collagen type I (Sigma-Aldrich, St. Louis, MO) at $2 \times 10^5$ cells per well and allowed to recover overnight after harvesting. The activities of hybridoma supernatants or purified mAbs were determined by application to cells at serial dilutions. Colo 205 cell adhesion activation was determined by the extent of morphological change to compact epithelial appearance, and the degree of activation was estimated by eye.

Live-cell ELISA
Tissue culture 96-well plates were precoated with 5 μg/ml of rat-tail collagen type I for 2 h at 37°C for seeding of Colo 205 cells or used

Agilent Technologies, Santa Clara, CA. The cDNAs for epitope mapping were cloned either into the pCS2 vector containing a C-terminal FLAG tag (Kim et al., 2009) for expression in the wheat germ in vitro system or into the pEE14 vector with a C-terminal Fc tag for transient transfection in Chinese hamster ovary cells. In the case of Chinese hamster ovary expression, the expressed proteins were purified from the cell culture media using protein A agarose.

Colo 205 cell activation assay
For the activation assay, Colo 205 cells were seeded on 96-well plates precoated with rat-tail collagen type I (Sigma-Aldrich, St. Louis, MO) at $2 \times 10^5$ cells per well and allowed to recover overnight after harvesting. The activities of hybridoma supernatants or purified mAbs were determined by application to cells at serial dilutions. Colo 205 cell adhesion activation was determined by the extent of morphological change to compact epithelial appearance, and the degree of activation was estimated by eye.

Live-cell ELISA
Tissue culture 96-well plates were precoated with 5 μg/ml of rat-tail collagen type I for 2 h at 37°C for seeding of Colo 205 cells or used
without precoating for other cell types. Cells were seeded at densities close to confluence, kept overnight to allow cadherin cell surface levels to recover, and incubated in duplicates (or greater multiples) with serial dilutions of hybridoma supernatants or purified mAbs in culture medium for 1 h at 37°C. After washing three times with culture medium, cells were incubated with horseradish peroxidase (HRP)–conjugated goat anti–mouse IgG (Bio-Rad, Hercules, CA) for 1 h at 37°C and then washed three times. Amount of binding was detected with 3,3′,5,5′-tetrakis(methylbenzidine substrate (Sigma-Aldrich). In some cases secondary goat anti–mouse IgG1 isotype antibodies labeled with Alexa 488 fluorescent dye were used. The significance of the difference between the curves was determined using two-way A (two-factor analysis of variance [ANOVA]), where two factors considered were “–/+ activation” (column factor) and “concentration” (row factor). For all curves analyzed, an interaction between these two parameters was not significant (cell activation and antibody concentration were independent), whereas the row factor was significant (good dose dependence). Column factor p values that considered were “–/+ activation” (column factor) and “concentration” (row factor). For all curves analyzed, an interaction between these two parameters was not significant (cell activation and antibody concentration were independent), whereas the row factor was significant (good dose dependence). Column factor p values that considered were “–/+ activation” (column factor) and “concentration” (row factor). For all curves analyzed, an interaction between these two parameters was not significant (cell activation and antibody concentration were independent), whereas the row factor was significant (good dose dependence). Column factor p values that considered were “–/+ activation” (column factor) and “concentration” (row factor). For all curves analyzed, an interaction between these two parameters was not significant (cell activation and antibody concentration were independent), whereas the row factor was significant (good dose dependence). Column factor p values that considered were “–/+ activation” (column factor) and “concentration” (row factor). For all curves analyzed, an interaction between these two parameters was not significant (cell activation and antibody concentration were independent), whereas the row factor was significant (good dose dependence). Column factor p values that.

**Table 2**: Summary of roles of individual p120-catenin phospho-Ser/Thr sites in adhesion activation of Colo 205 cells.

| Residue | Dephosphorylation during mAb-induced activation | S,T→A activation | S,T→E blocks mAb-induced activation |
|---------|-----------------------------------------------|------------------|------------------------------------|
| S252    | ND                                            | –                | –                                  |
| S268    | +                                             | +                | +                                  |
| S288    | +                                             | +                | +                                  |
| T310    | +                                             | ND               | ND                                 |
| S312    | ND                                            | +/-              | +/-                                |
| T916    | –                                             | ND               | ND                                 |

Sites involved in adhesion regulation determined by single mutations (see Supplemental Figures S6 and S7) and sites shown to be dephosphorylated during adhesion activation determined using phosphospecific antibody staining (Figure 8) are shown. +, observed effect; –, no effect; ND, not determined.

Structural modeling

Structural modeling was done using PyMOL 1.3 software (DeLano Scientific, Palo Alto, CA). For E-cadherin modeling, the mouse E-cadherin 3Q2V.pdb file was used (Harrison et al., 2010); for C-cadherin, 1L3W.pdb was used (Boggon et al., 2002).

Electrophoresis and Western blotting

SDS–PAGE was performed using standard procedures. For basic-native gels a protocol from Mario Lebendiker (Wolfson Center for Applied Structural Biology, Hebrew University of Jerusalem, Jerusalem, Israel) was used. Briefly, SDS and reducing agents were excluded from all solutions; otherwise general Laemmli electrophoresis protocol was applied. Before loading, the gels were prerun for 15–20 min. Western blot analysis was performed using standard procedures. Blots were developed with HRP-labeled secondary antibody and further processed with a Fujifilm LAS-3000 instrument (Fujifilm, Tokyo, Japan). Alternatively, blots were developed using IRDye 800CW goat anti-mouse (926-32210; LI-COR Biosciences, Lincoln, NE) and IRDye 680 goat-anti-rabbit (926-32221; LI-COR Biosciences) secondary antibodies and processed in a LI-COR Odyssey infrared imaging system.
Immunofluorescence, confocal, and bright-field imaging

For immunofluorescence, cells were grown on glass coverslips coated with 5 μg/ml of either fibronectin (for MDA-MB-231) or rat-tail collagen (Colo 205), fixed with 4% PFA for 10 min at room temperature, and permeabilized with 0.25% Triton X-100. For secondary antibodies, goat anti-mouse IgG (H+L)–Alexa 488 (A11001), goat anti–mouse IgG2b–Alexa 488 (A21141), IgG2b–Alexa 546 (A21143), IgG2a–Alexa 488 (A21131), and IgG2a–Alexa 546 (A21133), and goat anti–rabbit IgG–Alexa 546 (A11010) (all Invitrogen, Carlsbad, CA) were used in different combinations. Nuclei were counterstained with To-Pro-3 iodide (T3605; Invitrogen). All images were acquired at room temperature. For confocal imaging, coverslips were mounted in ProLong Gold anti-fade reagent (P36930; Invitrogen). Images were acquired using an Eclipse TE2000 confocal microscope (Nikon), PlanApo 60×/1.40 objective lens with immersion oil, high-quality charge-coupled device camera, and EZ-C1 software (Nikon). For general immunofluorescence, images were acquired using an IX-71 fluorescence microscope (Olympus, Center Valley, PA), UPlanFL N 10×/0.30 objective lens, a digital charge-controlled device camera C10600-10B (Hamamatsu, Hamamatsu, Japan), and SlideBook 5.0 Software (Intelligent Imaging Innovations, Denver, CO). Phase contrast and bright-field images were acquired using an Axiovert inverted microscope (Zeiss), LD-PlanApo 20×/0.3 or AchroPlan 4×/0.1 objective lenses, and PowerShot G6 camera (Canon, Lake Success, NY).

Mouse p120-catenin retroviral constructs, infections, and human p120 knockdown

Retroviral constructs in the pLZRS Neo vector, including mouse wild-type p120 catenin isoform 3A and several mutants thereof (S252A, S252E, S268A, S268E, S288A, S288E, S312A, S312E, 65,T→A) were kind gifts from Albert Reynolds (Ireton et al., 2002; Xia et al., 2006). The 65,T→A mutant harbors S252A, S252E, S288A, T310A, S312A, and T916A mutations. Additional mutations in mouse p120 catenin isoform 3A were introduced using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) and cloned into the pLZRS Neo vector. The 45,S→E mutant harbors S268E, S288E, T310E, and S312E mutations. All retroviral vector and viruses were produced using the Phoenix retrovirus producer cell line (Garry P. Nolan, Stanford University) according to the Nolan lab protocol. Cells were infected with respective retroviruses by spinoculation in six-well tissue culture plates at 1800 × g for 2 h at 33°C and selected with 1 mg/ml neomycin for 10 d. In the case of Colo 205 cells, multiple infections with the same virus were performed to achieve the desired expression level. Mock-treated cells were infected with retrovirus containing empty vector and subjected to selection as for the other lines. Mouse p120 catenin expression levels were estimated by Western blot analysis using mouse p120–specific mAb 8D11 (Wu et al., 1998), a kind gift from Albert Reynolds. Human p120 catenin knockdown was achieved by electroporation of infected cells with human p120–specific siRNA (Davis et al., 2003) using Amaxa Nucleofector (Lanza, Basel, Switzerland) according to the manufacturer’s instructions.

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