# Roles for E-cadherin cell surface regulation in cancer

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**ABSTRACT** **The loss of E-cadherin expression in association with the epithelial–mesenchymal transition (EMT) occurs frequently during tumor metastasis. However, metastases often retain E-cadherin expression, an EMT is not required for metastasis, and metastases can arise from clusters of tumor cells. We demonstrate that the regulation of the adhesive activity of E-cadherin present at the cell surface by an inside-out signaling mechanism is important in cancer. First, we find that the metastasis of an E-cadherin–expressing mammary cell line from the mammary gland to the lung depends on reduced E-cadherin adhesive function. An activating monoclonal antibody to E-cadherin that induces a high adhesive state significantly reduced the number of cells metastasized to the lung without affecting the growth in size of the primary tumor in the mammary gland. Second, we find that many cancer-associated germline missense mutations in the E-cadherin gene in patients with hereditary diffuse gastric cancer selectively affect the mechanism of inside-out cell surface regulation without inhibiting basic E-cadherin adhesion function. This suggests that genetic deficits in E-cadherin cell surface regulation contribute to cancer progression. Analysis of these mutations also provides insights into the molecular mechanisms underlying cadherin regulation at the cell surface.**

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## INTRODUCTION

E-cadherin is a well-known tumor suppressor protein, and the loss of its expression in tumor cells, in association with the epithelial–mesenchymal transition (EMT), occurs frequently during tumor progression and metastasis (Cano *et al.*, 2000; Yang and Weinberg, 2008; Nieto, 2011; Valastyan and Weinberg, 2011; Huang *et al.*, 2012). The resulting loss of cell–cell adhesion and cell junctions mediated by E-cadherin homophilic binding is believed to allow cells to dissociate from the primary tumor, invade surrounding tissues, and migrate to distant sites. However, carcinomas and distal metastases often retain E-cadherin expression (Yang and Weinberg, 2008; Shamir *et al.*, 2014), and the EMT is not required for metastasis to occur (Lou *et al.*, 2008; Hollestelle *et al.*, 2013; Fischer *et al.*, 2015; Whittle and Hingorani, 2015; Zheng *et al.*, 2015); in fact, clusters of tumor cells have been found to give rise to mammary metastases (Aceto *et al.*, 2014; Cheung *et al.*, 2016). Moreover, E-cadherin is involved in collective cell behaviors that facilitate invasion and metastasis (Cheung *et al.*, 2013; Shamir *et al.*, 2014). Changes in E-cadherin function other than complete loss of expression may be important for these processes.

We showed that E-cadherin adhesive activity can be regulated at the cell surface by an inside-out signaling mechanism probably involving allosteric regulation of the homophilic adhesive bond, analogous to integrin regulation (Petrova *et al.*, 2012; Shashikanth *et al.*, 2015; Maiden *et al.*, 2016). Moreover, this form of surface regulation participates in cell rearrangements and tissue morphogenesis, as in C-cadherin regulation during *Xenopus* gastrulation (Brieher and Gumbiner, 1994; Zhong *et al.*, 1999) and E-cadherin regulation in epithelial cell migration and branching (Petrova *et al.*, 2012). We hypothesize that similar mechanisms of E-cadherin regulation underlie changes in adhesion occurring in cancer cells.

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Abbreviations used: 4T1-hE, 4T1 cell line expressing human E-cadherin; 6S>A, form of p120 with six serine-to-alanine mutations; CLP, cleft lip and palate; colo-hE-shRNA, colo205 cells with shRNA-mediated depletion of E-cadherin expression; EC, extracellular cadherin domain; EMT, epithelial–mesenchymal transition; FACS, fluorescence-activated cell sorting; HDGC, hereditary diffuse gastric cancer; mAb, monoclonal antibody; shRNA, short hairpin RNA; WT, wild type.

In this study, we took two approaches to investigate whether regulation of the adhesive activity of E-cadherin at the cell surface is important for tumorigenesis or tumor metastasis. In one, we asked whether metastasis of an E-cadherin–expressing mammary cell line to the lung depends on reduced E-cadherin adhesive function at the cell surface. To do so, we used an activating monoclonal antibody (mAb) to E-cadherin that we developed and characterized in a previous study (Petrova *et al.*, 2012) to keep the E-cadherin on the surface of these cells in a high adhesive state and determined whether it has any effect on the extent of metastasis.

We also undertook a series of experiments to explore whether cancer-promoting missense mutations in the E-cadherin gene affect the mechanism of cell surface regulation. Germline mutations in CDH1 (E-cadherin) are known to be a major causal factor in hereditary diffuse gastric cancer (HDGC), with ~30% of all patients harboring such a mutation (Guilford *et al.*, 1998; Suriano *et al.*, 2003; More *et al.*, 2007; Corso *et al.*, 2012; Carvalho *et al.*, 2013). Although the majority of HDGC mutations are nonsense mutations leading to truncation or absence of the E-cadherin protein, ~20% are missense mutations leading to changes in the amino acid sequence. Missense mutations in E-cadherin are also believed to contribute to cleft lip and palate (CLP) birth defects in some families (Carvalho *et al.*, 2013; Vogelaar *et al.*, 2013). Several somatic missense mutations associated with breast cancer are also reported in the The Cancer Genome Atlas (TCGA) database, although compared with the HDGC mutations, it is less clear whether they are causal. The HDGC and CLP mutations occur throughout different regions of the E-cadherin extracellular domain and also in the cytoplasmic domain. The mutations in the extracellular domain are not in residues known to mediate the homophilic binding mechanism. Although some very cursory studies suggested that these might be completely defective in cell adhesion (Suriano *et al.*, 2003; More *et al.*, 2007; Corso *et al.*, 2012), we hypothesize that some may instead affect the mechanism of inside-out regulation. Elucidating how these mutations affect regulation of E-cadherin adhesive activity can provide insights not only into the ways in which E-cadherin controls tumorigenesis but also into the molecular mechanisms underlying cadherin regulation at the cell surface.

## RESULTS

### Role of E-cadherin activity state in metastasis

We hypothesize that the ability of E-cadherin–expressing tumor cells to metastasize is due to a down-regulation of the adhesive activity state of E-cadherin at the surface rather than its amount. To test this hypothesis, we asked whether enhancement of E-cadherin adhesive activity by treatment with activating mAbs could affect the extent of metastasis of E-cadherin–positive cells. We used the 4T1 mouse mammary cell line, which rapidly metastasizes from mammary gland to lung despite high levels of E-cadherin expression and very epithelial-like characteristics and seems to metastasize without undergoing an EMT (Lou *et al.*, 2008).

Because our E-cadherin–activating mAbs were generated in mice, they do not recognize mouse E-cadherin (Petrova *et al.*, 2012). Therefore we generated a 4T1 cell line expressing human E-cadherin (4T1-hE) at a level very similar to endogenous mouse E-cadherin (Supplemental Figure 1A); these cells respond to E-cadherin–activating mAb treatment by the criteria of an adhesion flow assay (Supplemental Figure 1B). We confirmed in preliminary experiments by histological examination that these cells metastasize to lung similarly to parental 4T1 cells in both immunodeficient SCID mice and BALB/c mice (Supplemental Figure 1C). We chose to use the BALB/c model because it is the more natural condition, and the lung metastases formed numerous smaller masses, in contrast to one huge mass in the lungs in SCID mice, and would more likely reveal quantitative changes in the number of metastases.

To determine the effects of activating mAbs on metastasis, we injected 4T1-hE cells orthotopically into the mammary fat pad of BALB/c mice. Beginning at 3 d after inoculation, mice were treated twice weekly (via intraperitoneal injection) with either 19A11-activating mAb or a control neutral mAb that binds well to human E-cadherin at the surface but does not activate adhesion or block adhesion (Petrova *et al.*, 2012). There was no detectable difference in the growth in size of the primary orthotopic tumor in the mammary gland for activating versus neutral mAb (Figure 1). Preliminary experiments indicated that small metastases began to form at 20–25 d, with more robust metastases developing at 27 d (unpublished data). Because the growth of the primary

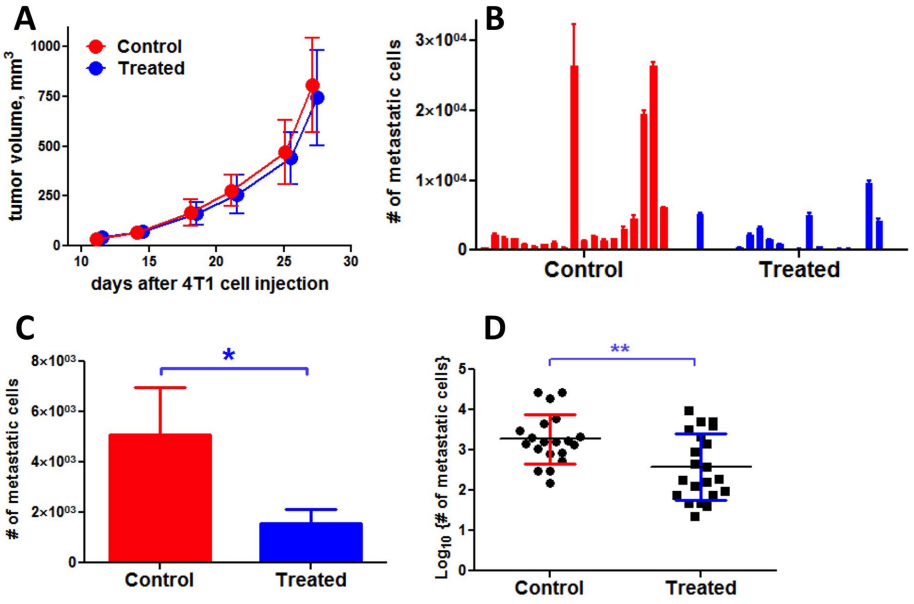


FIGURE 1: Activation of E-cadherin adhesion inhibits metastasis. Mouse epithelial 4T1Luc2 cells expressing human E-cadherin (4T1-hE) were injected into mammary fat pads of host mice. Beginning on day 3, animals received intraperitoneal injections of either control "neutral" E-cadherin–specific mAb 46H7 or E-cadherin–activating mAb 19A11 twice weekly until the end of the experiment. (A) Caliper measurements of the size of the primary tumor formed in the mammary glands showed no difference over time between control and activating mAb–treated groups. (B–D) Whole-lung qRT PCR analysis using a luciferase sequence expressed in 4T1Luc2 cells to count the 4T1-hE cells metastasized to lung at 27 d after injection. A calibration curve was used in which known numbers of 4T1-hE cells were mixed with lung homogenate. GAPDH was used as a housekeeping gene to normalize for tissue amount. (B) Data from individual animals. (C) Mann–Whitney *U* test was used to determine statistical difference between groups because the data in both groups did not show a Gaussian distribution according to the Kolmogorov–Smirnov normality test (*$p = 0.0147$). (D) Alternatively, data were transformed as $\log_{10}$ and analyzed by Student's *t* test (**$p = 0.004$).

| Number | Stain | Treatment | Slide number | Immunoreactivity |
|---|---|---|---|---|
| 1 | E-cadherin | Treated | M1.4 T1-b | 75% strongly positive |
| 2 | | | M4.4 T7-b | 50% strongly positive |
| 3 | | | M4.3 T6-b | 80% strongly positive |
| 4 | | | M2.2 T3-b | 90% strongly positive |
| 5 | | Control | M1.2 C2-b | 80% strongly positive |
| 6 | | | M5.1 C9 | 70% moderately positive |
| 7 | | | M5.3 C10-b | 90% strongly positive |
| 8 | Vimentin | Treated | M1.4 T1-a | 90% strongly positive |
| 9 | | | M2.2 T3-a | 90% strongly positive |
| 10 | | | M4.3 T6-a | 70–80% strongly positive |
| 11 | | | M4.4 T7-a | 80% moderately to strongly positive |
| 12 | | Control | M1.2 C2-a | 90% strongly positive |
| 13 | | | M2.3 C4 | 90% strongly positive |
| 14 | | | M5.3 C10-a | 90% moderately positive |
| 15 | Ki67 | Treated | M3.1 T4 | 33.5% positive cells |
| 16 | | | M4.2 T5 | 34.1% positive cells |
| 17 | | | M6.2T10 | 24.7% positive cells |
| 18 | | Control | M1.1 C1 | 26.8% positive cells |
| 19 | | | M2.4 C5 | 42.7% positive cells |
| 20 | | | M3.3 C6 | 40.5% positive cells |

Twenty slides were evaluated using immunohistochemistry for expression of E-cadherin, vimentin, and the proliferation marker Ki67. There were no marked differences between the activating mAb–treated (Treated) and neutral mAb–treated (Control) groups for the three evaluated immunohistochemical stains. In treated groups, the percentage immunoreactivity for E-cadherin ranged from 50 to 90%, whereas in the control group, it ranged from 70 to 90%. For vimentin, the treated and control groups showed 70–90 and 90% positive immunoreactivity, respectively. The Ki67 index (evaluated with Indica Labs CytoNuclear v1.5 algorithm) in the treated group ranged from 24 to 34% positive cells, whereas that in the control group ranged from 27 to 43%.

**TABLE 1:** Immunohistochemistry of 4T1 cell–derived tumors after mAb treatment.

tumor limited our experiment to 27 d (guidelines required killing the animals at this point), we collected most samples at this time point. Quantitative PCR of a gene uniquely expressed in the 4T1-hE cells (the synthetic firefly luciferase luc2 gene) was used to determine the number of cells that metastasized to the entire lung of each mouse. There was a clear decrease in the number of 4T1-hE cells metastasized to lung in mice treated with activating mAb relative to control neutral mAb (Figure 1). Samples from individual animals are shown in Figure 1B, and the population data are given in Figure 1, C and D. The differences were statistically significant using two calculations: a Mann–Whitney *U* test (Figure 1C, $*p = 0.0147$) and Student's *t* test after the data were transformed as $\log_{10}$ (Figure 1D, $**p = 0.004$). Thus, stimulating the activity state of E-cadherin on the cell surface inhibits the metastatic progression, suggesting that down-regulation of adhesion in these tumor cells contributes to their metastatic potential despite high levels of E-cadherin expression.

Although activating mAbs had no effect on the growth in size of the primary orthotopic tumor in the mammary gland, we examined the primary tumors for possible changes related to their potential to metastasize (Table 1 and Supplemental Figure 2). There was no quantitative difference in the number of cells expressing the proliferation marker Ki67, consistent with the lack of effect on tumor size. Both control and activating mAb–treated tumors expressed high levels of E-cadherin, which was concentrated at regions of cell–cell contact, indicating that cells exhibited epithelial properties in both cases, just as they do in cell culture (Supplemental Figure 1A). There was also no obvious effect on the percentage of cells expressing vimentin, a commonly used marker for the EMT; in fact, a high percentage of cells expressed vimentin in both cases. Although a previous publication reported that tumors arising from 4T1 cells did not stain strongly for vimentin, it did show that cultured 4T1 cells express moderate amounts of vimentin using biochemical assays (Lou *et al.*, 2008). Although 4T1 cells in the tumors retain strong epithelial properties and E-cadherin expression in cell junctions, they also exhibit some mesenchymal characteristics that could be important for metastasis.

### Effects of germline HDCG mutations on E-cadherin adhesion and activation

Numerous missense germline mutations in the E-cadherin gene have been implicated in the pathogenesis of diffuse gastric cancer (Carvalho *et al.*, 2013). Several somatic missense mutations associate with breast cancer are also reported in the TCGA database, although compared with the HDGC mutations, it is less clear whether they are causal; therefore we decided to focus on the well-documented causal HDCG germline mutations. Many cause amino acid substitutions at different regions of the extracellular domain (Table 2; also see Figure 6 later in the paper), and most are not in regions believed to have a direct role in cadherin homophilic binding interactions per se. Although a few of these have been suggested to cause defects in adhesion as a result of cursory assays (Suriano *et al.*, 2003; Brooks-Wilson *et al.*, 2004; More *et al.*, 2007), we sought to test rigorously whether they still mediate homophilic adhesion assay and, if so, how well. We used a well-established quantitative adhesion assay that

| Mutation (pre-proprotein) | Mutation (mature protein) | Structural location | Function[a] |
|---|---|---|---|
| HDGC germline mutations | | | |
| P172R | P18 | Free loop, EC1 | Nonadhesive |
| R224C | R70 | Free loop, close to Ca-binding site, EC1–EC2 interface; important for recognition by activating mAbs | Partial |
| G239R | G85 | Free loop, close to the top of EC1 | Uncoupled |
| D244G | D90 | Free loop, close to the top of EC1 | No activation |
| S270A | S116 | Structured, b-strand, close to the bottom of EC2 | No activation |
| A298T | A144 | Structured, helix, close to Ca-binding site, EC1–EC2 interface | WT |
| T340A | T186 | Structured, end of b-strand, close to Ca-binding site, EC2–EC3 interface | No activation |
| P373L | P219 | Free loop, close to Ca-binding site, EC2–EC3 interface | Partial |
| P377R | P223 | Free loop, EC2–EC3 interface | WT |
| W409R | W255 | Free loop, close to Ca-binding site, EC2–EC3 interface | WT |
| V487A | V333 | Free loop, close to Ca-binding site, EC3–EC4 interface | Constitutive |
| L583R | L429 | Structured, b-strand, EC4 | Partial |
| A592T | A438 | Free loop, close to Ca-binding site, EC4–EC5 interface | WT |
| T599S | T445 | Free loop, EC5 | Partial |
| A617T | A463 | Free loop, close to Ca-binding site, EC4–EC5 interface | Partial |
| A634V | A480 | Free loop, bottom of EC5 | Constitutive |
| CLP mutation | | | |
| D370Y | D216 | Free loop, Ca-coordinating amino acids, EC2–EC3 link | No activation |
| Experimental mutations | | | |
| W156A | W2 | Free N-terminus, EC1 | Nonadhesive |
| K168E | K14 | Free loop, close to Ca-binding site, EC1–EC2 interface, X-dimer mutant | Nonadhesive |
| EED > AAA | 608,9,10 | Cytoplasmic tail, p120-free mutant | Constitutive |

[a]Nonadhesive, exhibits absolutely no adhesion activity when expressed in either CHO cells or colo205 cells even when exposed to activating stimuli; WT, adhesion activation like WT E-cadherin; No activation, not activatable by any stimuli in colo205 cells despite having strong adhesion in CHO cells; partial, partially activatable by all stimuli in colo205 cells, with normal adhesion in CHO cells; uncoupled, activated from the outside by activating mAbs but not from intracellular stimuli; constitutive, constitutively activated in colo205 cells even in the absence of any activating stimuli.

**TABLE 2:** Disease-related and experimental human E-cadherin mutations.

determines the strength of cell adhesion to a substrate coated with purified E-cadherin protein (Yap *et al.*, 1997; Chappuis-Flament *et al.*, 2001). Each of the mutant human E-cadherin proteins was expressed in nonadhesive CHO cells that completely lack cadherin expression. Because we wanted to focus on the adhesive activity of these proteins at the cell surface, we analyzed expression levels by flow cytometry (Supplemental Figure 3A) to ensure that any substantial differences in adhesion could not be attributable to variations in the levels at the cell surface. If expression differed from WT or other controls, we used fluorescence-activated cell sorting (FACS) to select cells expressing similar levels at the surface. We did not notice any large differences in overall levels of expression by Western blotting (unpublished data).

The strength of adhesion of CHO cells expressing mutant E-cadherins was determined by the shear force required to detach the cells from the E-cadherin–coated surface. Adhesion was strongly abrogated by only one of these mutations, P172R (P18 in mature protein; Figure 2C), similar to experimental mutations known to disrupt formation of the homophilic bond (Boggon *et al.*, 2002; Harrison *et al.*, 2010)—W2A and K14E of the mature protein (W156A and K168E, respectively, in Figure 6 later in the paper). The other mutations exhibited adhesion strengths either similar to that of WT E-cadherin (Figure 2A) or a little less strong than that of WT, albeit still quite significant compared with adhesion-dead mutations (Figure 2B). Therefore most of the mutant proteins still exhibited good levels of adhesion activity in CHO cells.

We therefore tested whether these mutations affected the regulation of adhesion rather than the basal adhesive function of the molecule, using colo205 cells, which exhibit a dramatic regulation of adhesive states, with activation of cell adhesion depending on treatment with various stimuli (Aono *et al.*, 1999; Petrova *et al.*, 2012; Shashikanth *et al.*, 2015; Maiden *et al.*, 2016). These cells provide a unique experimental model for cadherin biology, analogous to the integrin activation in platelets or leukocytes. In most circumstances, cadherin regulation is subtle and occurs in continuously adhesive cells and therefore is harder to dissect; colo205 cells behave like the compaction of the early mouse embryo, in which adhesion, junction formation, and epithelialization occur rapidly in response to a stimulus (Johnson, 2009). To enable analysis of the exogenously introduced E-cadherin mutants, we first knocked down endogenous E-cadherin expression using short hairpin RNA (shRNA). The resulting colo205-derived clonal

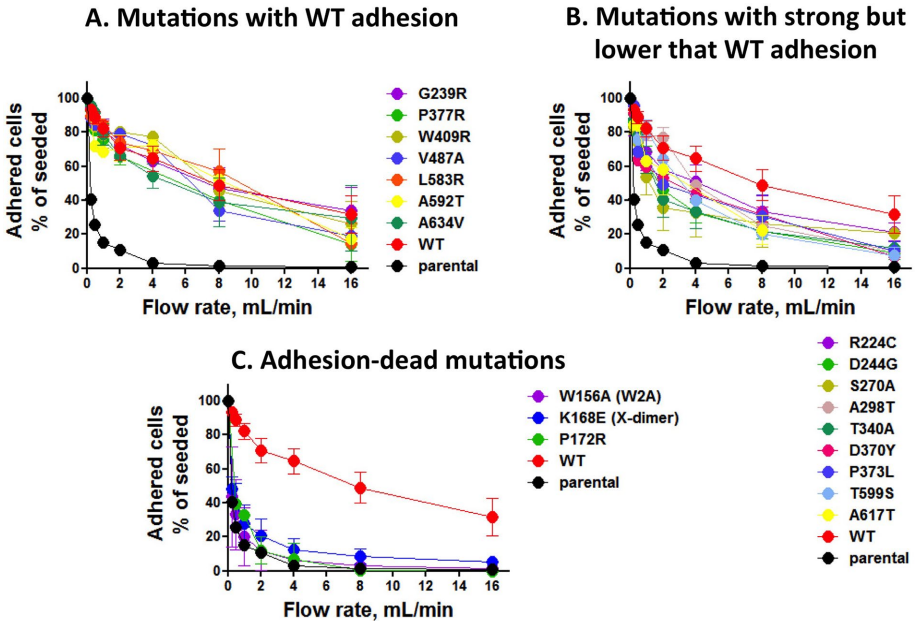


FIGURE 2: Most disease-related mutations in human E-cadherin allow significant basal adhesion activity. The various E-cadherin mutant proteins were expressed in CHO cells by lentiviral infection, and expression levels were verified by flow cytometry. Adhesion strength was evaluated using increasing laminar flow to determine the force required to detach cells. (A) Mutants with the same strength of adhesion compared with WT E-cadherin. (B) Mutants demonstrating strong adhesion but a bit lower than that of WT E-cadherin. (C) Mutants showing no detectable E-cadherin–mediated adhesion. Only the P172R is a naturally occurring cancer-associated mutation; the others are known from published structural and experimental investigations (W156A = W2A strand dimer mutant; K168E = K14E X-dimer mutant). Error bars indicate the SD of several independent experiments ($N = 4$).

cell line (colo205 cells with shRNA-mediated depletion of E-cadherin expression [colo-hE-shRNA]) expressed very low levels of E-cadherin. Adhesion of colo-hE-shRNA cells could not be activated by any treatment (Figure 3A; unpublished data), but reexpression of WT E-cadherin reconstituted adhesion activation by activating mAbs compared with control neutral mAbs, which bind but do not activate (Figures 3–5 and Supplemental Figures 4–8). We previously showed that E-cadherin–mediated adhesion in colo205 cells could also be activated intracellularly by dephosphorylation of p120-catenin (Petrova *et al.*, 2012) and that p120-catenin dephosphorylation and adhesion activation can be induced by treatment with LiCl (GSK3b inhibitor) or nocodazole to disassemble microtubules (Shashikanth *et al.*, 2015; Maiden *et al.*, 2016). WT E-cadherin expressed in colo-hE-shRNA cells can also be activated by these treatments (Figures 3–5 and Supplemental Figures 4–8).

Each mutant E-cadherin protein was expressed in colo-hE-shRNA cells and compared with WT E-cadherin. Because we wanted to focus on the adhesive activity of these proteins at the cell surface, we made considerable effort to ensure similar levels of cell surface expression of all mutant proteins compared with WT using flow cytometry, and, when necessary, FACS was used to select for a relatively homogeneous distribution of surface expression (Supplemental Figure 3B). Expression of each mutant was even assessed immediately before each experiment using a simple desktop flow cytometer. There were no obvious changes in overall expression levels via Western blotting (unpublished data). Both activation of adhesion from the outside by activating mAbs and activation from the inside by LiCl treatment (and sometimes nocodazole) were assessed by the formation of compacted cell aggregates using microscopic observation (Figures 3–5 and Supplemental Figures 4–8) and confirmed for the entire population of cells in the sample by a quantitative assay (Figure 3; see later discussion). Unsurprisingly, expression of the adhesion-dead mutations, either known experimental W2A (W156A) or K14E (K168E) mutation or the P172R HDGC mutation, were not activatable by any treatment (Supplemental Figure 3). This is consistent with these mutations inhibiting the formation of the homophilic adhesive bond. This was certainly expected for the W2A mutation, which is a key part of the homophilic bond (Boggon *et al.*, 2002). It also indicates that activation cannot occur without the formation of the X-dimer structure, which is believed to be intermediate in the molecular pathway for homophilic bond formation and is blocked by the K14E mutation (Harrison *et al.*, 2010).

Many of the HDGC mutations interfere with the activation process even though they do not greatly inhibit basal homophilic adhesion binding activity (Figure 2). The most striking are mutations D244G, S270A, and T340A (D90, S116, and T186 in mature protein), which cannot be activated by either activating mAbs or LiCl treatment (Figure 4 and Supplemental Figure 5). A mutation known to give rise to cleft lip and palate (CLP) in humans, D370Y (D216 in mature protein) similarly inhibits activation of adhesion (Figure 2) despite mediating basal cell adhesion (Figure 2). Several other HDGC mutations partially inhibit activation by both mAbs and LiCl treatment; they are weakly activatable but detectably less so than WT E-cadherin (Figure 4 and Supplemental Figure 6; see Figure 3E for quantitation). These include R224C, P373L, L583R, T599S, and A617T (in mature protein, R70, P219, L429, T445, and A463). Except for R224C (see later discussion), none of the residues altered by mutations that affect activation is part of the epitope recognized by the activating mAb (Petrova *et al.*, 2012), and the activating mAb recognizes all of the mutated proteins on the cell surface by flow cytometry and/or immunofluorescence staining (unpublished data). Therefore several mutations in E-cadherin that contribute to the development of HDGC and CLP inhibit the activation of adhesion, selectively interfering with a step in E-cadherin activation rather than basic adhesive function.

A particularly interesting mutation is HDGC mutation G239R (G85 in mature protein), which appears to uncouple different modes of activation (Figure 5). Just as in WT, E-cadherin adhesion mediated by this protein is high in CHO cells (Figure 2A) and strongly activated by activating mAbs, as well as by treatment of colo205 cells with low levels of trypsin (Figure 5, top), as previously described (Aono *et al.*, 1999; Petrova *et al.*, 2012). However, none of the stimuli believed to act intracellularly via p120-catenin dephosphorylation—nocodazole, LiCl, or the broad-spectrum kinase inhibitor staurosporine—activates adhesion of the cells expressing this mutation. Indeed, unlike parental colo205 cells or colo205 cells expressing WT hE-cadherin, colo205 cells expressing G239R E-cadherin cannot be activated by expression of a phosphorylation-mutant form of p120-catenin (bottom), a p120-catenin form with six serine-to-alanine mutations (6S>A; Petrova *et al.*, 2012). These findings suggest

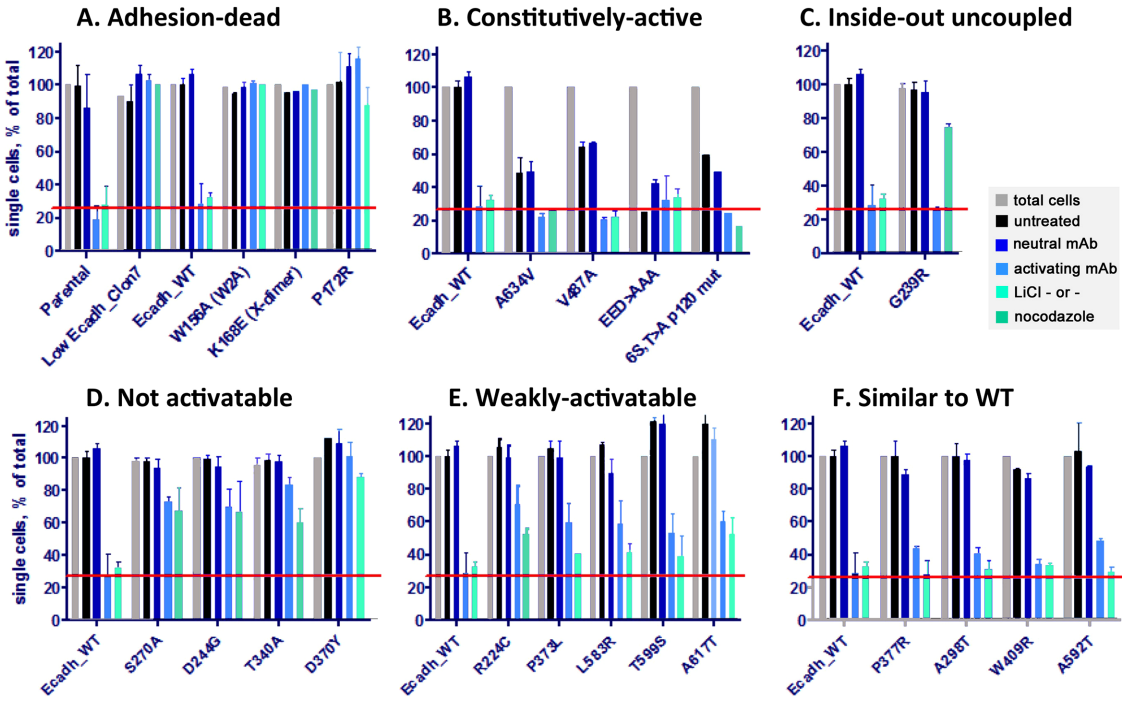


FIGURE 3: Quantification of E-cadherin–mediated adhesion activation with a cell counting assay. Experiments shown in Figures 4 and 5 and Supplemental Figures 4–8 were also analyzed by a quantitative cell aggregation assay (from Figure 5, the mAb- and LiCl-treated samples were used). Mutants were grouped according to demonstrated adhesion activation properties associated with each previous figure. Cells and clumps were lifted from the dish by gentle pipetting, and cell aggregates were filtered out using a flow cytometry cell strainer. Adhesion activation was assessed by counting the loss of single cells using a bench-top flow cytometer. To normalize the percentage of single cells to the total cell number, the total cell number was determined by counting cells harvested by trypsin/EDTA solution, which completely dissociates all cell aggregates (gray bars). Treatment of each mutant cadherin–expressing cell is shown in the same sequence: untreated (black), neutral mAb (dark blue), activating mAb (bright blue), and either LiCl (turquoise) or nocodazole (blue-green). Normal adhesion activation is demonstrated by parental colo205 cells in A and by WT E-cadherin–expressing cells in all graphs. At the other extreme, completely inactive cadherins are shown by the W156A (W2A strand dimer) and K168E (X-dimer) mutations, which are known to abolish adhesion. Error bars indicate SD of several independent experiments ($N = 3–6$).

that the G239R does not affect the ability of the extracellular domain to undergo changes necessary for adhesion activation but instead renders the E-cadherin resistant to intracellular signaling events that normally cause activation.

Several HDGC missense mutations have no detectable effect on adhesion activation in these assays (Supplemental Figure 7). A298T, P377R, W409R, and A592T (in mature protein, A144, P223, W255, and A438) behave like WT E-cadherin when treated with activating mAbs, LiCl, or nocodazole (unpublished data). Either their effects are too subtle to detect with these assays or they affect HDGC in a different way.

Surprisingly, two mutations, V487A and A634V (V333 and A480 in mature protein), appear to constitutively activate adhesion (Supplemental Figure 8). Their expression in colo-hE-shRNA cells leads to formation of compact aggregates independent of any antibody or inhibitor treatment, very similar to what we observed previously to result from expression of 6S>A p120-catenin mutant in colo205 cells (Petrova *et al.*, 2012) or expression of E-cadherin harboring a mutation that uncouples it from p120-catenin binding (Maiden *et al.*, 2016; shown in Supplemental Figure 8 for comparison). These two HDGC mutations may either cause the E-cadherin ectodomain to adopt an activated state or conformation or block the ability of phosphorylated p120-catenin to negatively regulate the adhesive activity of E-cadherin. In any case, they add further evidence that disease mutations in E-cadherin affect its state of adhesion activity at the cell surface.

In most cases, the effects of mutations of the activatability of E-cadherin in colo205 cells were readily apparent using visual inspection of cell culture morphologies, although the weakly activatable mutations exhibiting partial effects were subtler (Figure 4 and Supplemental Figure 6). To ensure measurement of the entire population of cells in the sample, we also developed a quantitative assay that assesses the extent of aggregation of all of the cells in a culture (Figure 3). As with early quantitative adhesion assays (Takeichi, 1977, 1988; Yoshida-Noro *et al.*, 1984), we used the reduction in number of single cells or very small clusters as a measure of adhesion-mediated cell aggregation. Each sample of cells was gently lifted from the well of the dish (colo205s do not attach well to the plastic), large aggregates were removed with filters used to generate samples suitable for flow cytometry analysis, and the remaining particles (single cells or small clusters) were counted using a desktop flow cytometer. To determine the total numbers of cells present, a parallel well for each sample was harvested with trypsin-EDTA to completely dissociate clusters into single cells. As shown in Figure 3A, treatment of parental colo205 cells or colo205s expressing WT E-cadherin with activating mAbs and LiCl caused a

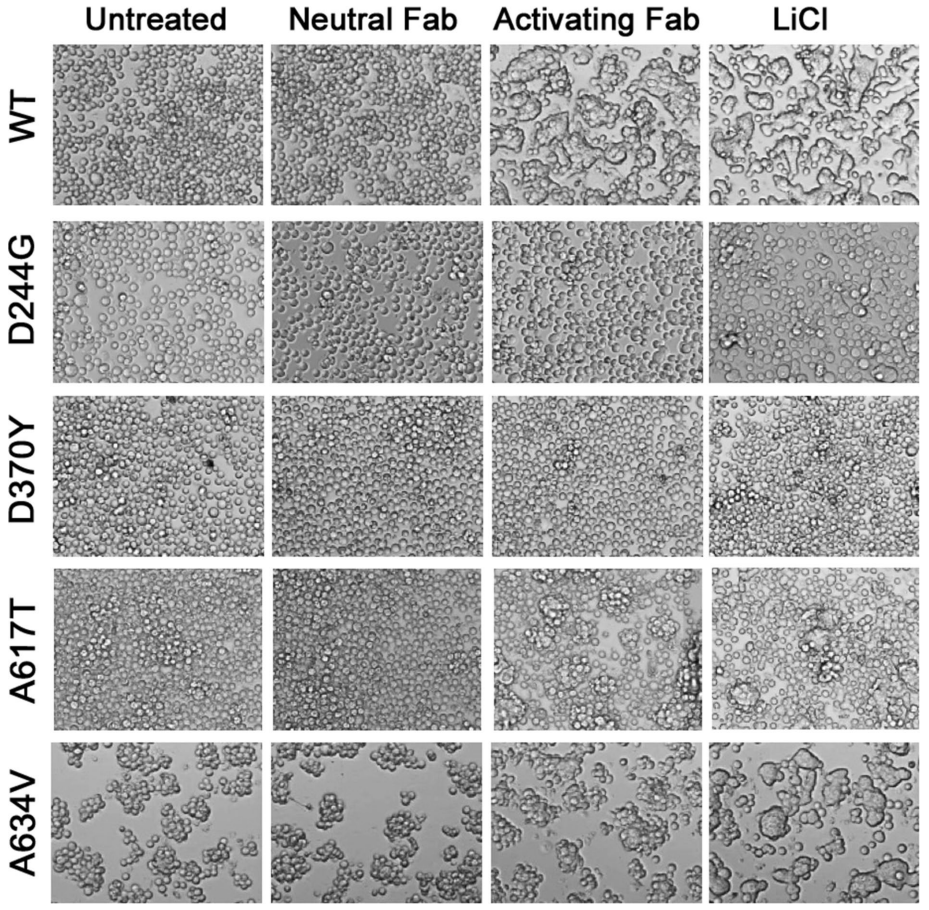


FIGURE 4: Effects of HDGC and CLP E-cadherin mutations on adhesion activation. Examples from each adhesion phenotype are shown; full data on all mutations in each category are shown in Supplemental Figures 4–8. WT, HDGC, and CLP (D370Y) E-cadherin mutants were expressed in colo-hE-shRNA cells by lentiviral infection, with comparable expression levels verified by flow cytometry. Cells were treated with E-cadherin–specific "neutral" 76D5 mAb or "adhesion-activating" 19A11 Fab fragments at 1 μg/ml for 5 h or with 60 mM LiCl for 1 h. Adhesion activation was assessed visually by extension of cell aggregation and right intercellular compaction and flattening. The activating Fabs and the LiCl treatment strongly activated adhesion. The D244G HDGC mutation and the D370Y CLP mutation failed to be activated by treatment with either activating Fabs or LiCl. The A617T HDGC mutation was partially activatable, and the HDGC mutation A634V was constitutively active when expressed in colo205 cells independent of any activating stimuli.

strong reduction in the number of single cells/small clusters compare to untreated or neutral mAb–treated controls. In contrast, colo205 cells depleted of E-cadherin (colo-hE-shRNA) or those expressing E-cadherin with adhesion-dead mutations (W2A/W156A, K14E/K168E, P172R) showed no reduction in single cells/small clusters when treated with activating mAbs or LiCl. These two extreme conditions demonstrate the validity of the assay for quantitating adhesion activation. This assay also provided quantitative evidence that V487A and A634V (V333 and A480) mutations constitutively activate adhesion, although they could be further activated by treatment with activating mAb or LiCl (Figure 3B). The G239R (G85) mutation is activatable by activating mAb but not LiCl (Figure 3C), whereas the D244G, S270A, and T340A (D90, S116, and T186) mutations were poorly activated by either treatment (Figure 3D). The weakly activatable mutants shown in Supplemental Figure 6 were quantitatively better activated by mAb or LiCl (Figure 3E) than the inactivatable mutants (Figure 3D) but not as well as WT E-cadherin or mutations with no detectable effects (Figure 3F). Overall, this quantitative assay confirmed what we found using visual observation of the morphology of colo205 cell colonies.

E-cadherin–mediated cell adhesion is often believed to affect tumor invasion by inhibiting cell migration away from the epithelial layer. Therefore we also asked how E-cadherin mutations affect cell migration, using a scratch wound–healing assay (Table 3 and Supplemental Figure 9). Only the adhesion-dead mutant, P172R, behaves like experimental adhesion-dead mutants (W2A, K14E) or no E-cadherin expression, as expected. All the mutant forms that support adhesion, fully or partially, inhibit migration of CHO cells to some extent compared with adhesion dead or no cadherin; some inhibit migration as much as WT E-cadherin, but others less so. It is possible that some of the variation is due to factors other than physical cell adhesion, such as signaling.

### Mapping mutations on E-cadherin three-dimensional structure

Mapping the mutations that affect adhesion activation onto the three-dimensional (3D) structure of the E-cadherin ectodomain reveals that they are distributed over many regions of the molecule (see Figure 6 and Table 1 for a description). The only residue whose mutation completely abrogates adhesive binding is P172/P18, which is located right near the K14 residue in the loop known to be involved in forming the X-dimer intermediate required for formation of the homophilic adhesive bond (Harrison *et al.*, 2010). Given that a loss of proline residue could alter the conformation of the polypeptide backbone of this loop, it is possible that the P18R mutation also affects X-dimer formation, thereby disrupting adhesive binding.

The G239R mutation (G85R in mature protein) that uncouples adhesion activation from intracellular triggers is located at the distal end of the distal extracellular cadherin domain 1 (EC1) of the cadherin molecule, well away from the transmembrane domain that might be expected to mediate this sort of coupling. Of interest, the G85 residue is observed in the *cis*-dimer interface of the crystal structure to hydrogen bond with T164 in EC2 of the other protomer (Harrison *et al.*, 2011). This raises the possibility that *cis*-dimerization plays a role in inside-out regulation. However, the other mutations that affect activation have not been implicated in dimerization, nor are other residues in the *cis*-dimer interface mutated in HDGC.

One of the four mutations that strongly inhibit activation by all treatments, D244G (D90 in mature protein), lies near residues in EC1 believed to be important for adhesion. Residue E89 forms a salt bridge with the N-terminus of the mature protein, which stabilizes W2-mediated strand exchange and plays a role in adhesion (Harrison *et al.*, 2005). Although not part of the *cis*-dimer interface observed in the crystal structure, it is close to residues that do participate in this interface (H79, V81, N84, G85; Harrison *et al.*, 2011),

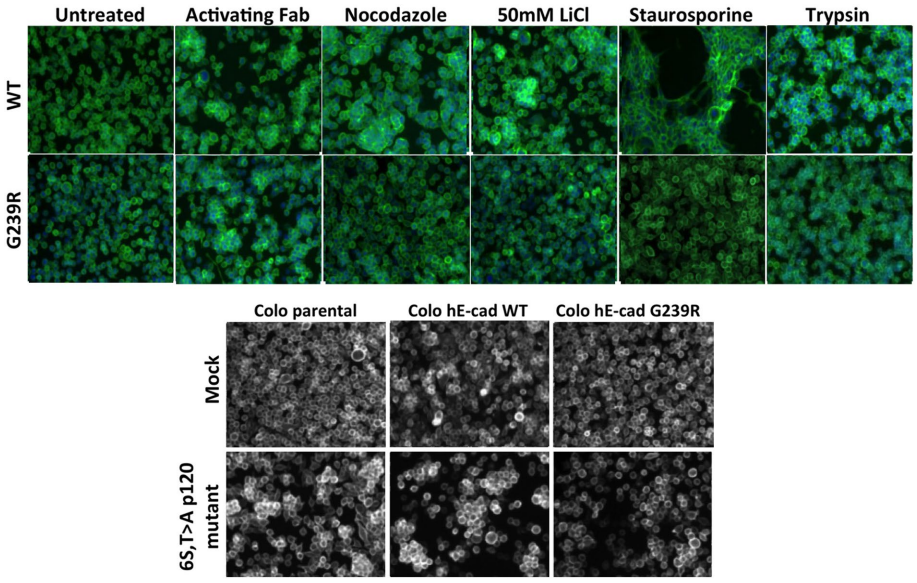


FIGURE 5: Uncoupling of extracellular and cytoplasmic activation of the HDGC-associated E-cadherin mutation G239R mutant. WT and G239R E-cadherin mutant were expressed in colo-hE-shRNA cells by lentiviral infection with similar expression levels as verified by flow cytometry. Top, G239R E-cadherin was strongly activated by 19A11 mAb Fab fragment or treatment for 30 min with 0.001% trypsin, similar to WT E-cadherin, but not by treatments that trigger activation of WT E-cadherin by intracellular stimuli via dephosphorylation of p120-catenin with nocodazole (10 μM for 1 h), LiCl (50 mM for 1 h), or staurosporine (7 nM for 5 h). Bottom, unlike WT E-cadherin, the G239R E-cadherin mutant was not activated by expression of the phosphodeficient 6S,T > A p120 catenin mutant, which was expressed by retroviral infection. All samples were immunostained for E-cadherin to better outline cell boundaries.

and so the D244G/D90G mutation could potentially affect *cis* interactions like the G239R/G85 mutation.

Three of the four mutations that strongly inhibit activation by all treatments are clustered near the interface between EC2 and EC3. The HDGC mutations S270A (S116 mature protein) and T340A (T186 mature protein) are present in structured β-strands close to the base (proximal side) of EC2, whereas the CLP mutation D370Y (D216 mature protein) is a calcium-coordinating residue at the EC2–EC3 link. This raised the possibility that the interface between EC2 and EC3 plays a particularly important role in activation and adhesion regulation. However, none of the mutations that only partially inhibit activation is located near this region but instead they are scattered over EC1, EC3, EC4, and EC5. This suggests that regions throughout the whole ectodomain may be involved in activation or adhesion regulation. Three of the five in this group—R224C (R70), P373L (P219), and A617T (A463)—are located near the calcium-binding sites at EC interfaces. However, several of the HDGC mutations with no detectable effect on adhesion or activation also lie near calcium-binding sites. The two mutations causing constitutive activation are located at the base (proximal side) of EC5, perhaps near the transmembrane domain (structure unknown), and in EC4 not far from its interface with EC3.

One of the partially activatable mutations R224C forms part of the conformational epitope recognized by the strongly activating mAbs near the calcium-binding site between EC1 and EC2 (Petrova *et al.*, 2012). These mAbs still appear to bind well to this mutant by criteria of immunofluorescence staining (Supplemental Figure 10). Moreover, R224C is also poorly activated by LiCl and not activated by another weakly activating mAb that recognizes a different epitope at the EC3–EC4 interface (unpublished data). Therefore it is possible that this mutation partially interferes with a local change at the EC1–EC2 interface that is induced by the strongly activating mAbs that bind to this region.

## DISCUSSION

Loss of E-cadherin expression is often associated with tumor metastasis, but it sometimes remains highly expressed on some distal metastases (Yang and Weinberg, 2008; Shamir *et al.*, 2014). Our findings provide a potential explanation for how tumor cells that retain high expression of E-cadherin can still metastasize. Because treatment of tumor-bearing animals with mAbs that activate E-cadherin adhesive activity significantly reduces metastasis, we conclude that physiological down-regulation of adhesion activity contributes to the metastatic process. Regulation of cadherin adhesive activity at the cell surface in response to growth factors has been shown to control tissue morphogenesis and epithelium formation (Brieher and Gumbiner, 1994; Zhong *et al.*, 1999; Petrova *et al.*, 2012). Our findings suggest that similar regulation of cell surface E-cadherin adhesive activity is important in cancer. The microenvironment of the tumor cells may produce signals that regulate E-cadherin activity, allowing for morphogenetic changes in migratory or invasive behavior that underlie metastasis.

Our findings also offer potential new explanations for the roles of E-cadherin mutations in human cancer. Although many cancer-associated changes in the E-cadherin gene lead to loss of E-cadherin protein expression (Cano *et al.*, 2000; Grady *et al.*, 2000) or

| Mutation | Adhesion assay | Migration |
|---|---|---|
| WT E-cadherin | WT | + |
| Parental | None | +++ |
| 156A (W2A) | None | +++ |
| K168E (K14E) | None | +++ |
| P172R | None | +++ |
| R224C | <WT | + |
| D244G | <WT | ++ |
| S270A | <WT | + |
| T340A | <WT | + |
| D370Y | <WT | ++ |
| P373L | <WT | + |
| T599S | <WT | + |
| A617T | <WT | + |
| G239R | WT | ++ |
| V487A | WT | + |
| A634V | WT | + |

TABLE 3: Effect of HDGC-associated E-cadherin mutations on cell migration in a wound closure assay.

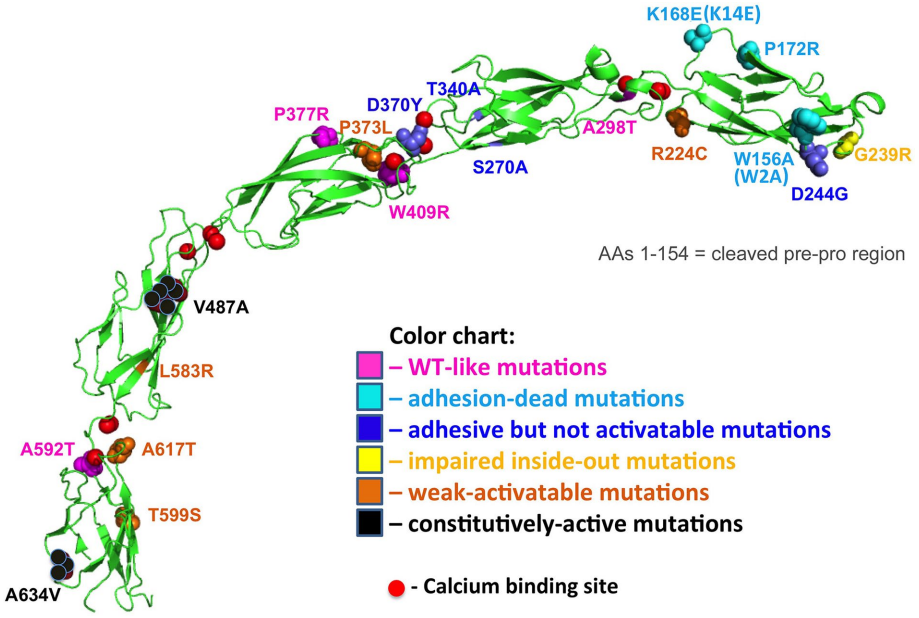


FIGURE 6: Structural modeling of various E-cadherin mutations with different effects on adhesion activation. Mouse E-cadherin 3Q2V.pdb file was used to show the positions of disease-related and experimental mutations of E-cadherin. Mutations located in free loops are shown as spheres; mutations in structural elements (helices, β-stands) are colored as a part of the polypeptide backbone. "WT-like" A298T, P377R, W409R, and A592T mutations are magenta, and "adhesion-dead" P172R, W156A (W2A), and K168E (K14E) mutants are cyan. Adhesive but not activatable D244G, S270A, T340A, and CLP-related D370Y mutants are blue, the G239R mutant with impaired inside-out signaling is yellow, weakly activatable R224C, P373L, L583R, T599S, and A617T mutants are orange, and constitutively active V487A and A634V mutants are black. Bright red spheres are calcium ions.

severe truncations in the protein, missense mutations also frequently occur (van Roy and Berx, 2008; Carvalho *et al.*, 2013). Because we find that many of the HDGC missense mutations selectively interfere with the regulation of E-cadherin adhesion in the colo205 model rather than basic adhesive activity, it is possible that molecular changes underlying E-cadherin regulation at the cell surface are needed in cancer cells to suppress cell behaviors that contribute to tumor development or progression, consistent with our finding that activation of adhesion with mAbs inhibits metastasis. This also implies that standard histopathological or genetic analyses of E-cadherin expression are probably not sufficient for evaluating the functional status of E-cadherin–mediated adhesion in tumors.

Analysis of the HDGC and CLP mutations also provides important insights into the mechanisms of cadherin regulation. These mutations are not found in residues believed to be essential for adhesive function, based on the known cadherin 3D structure. Indeed, we find that most of the E-cadherin germline missense mutations associated with HDGC and CLP retain basic adhesive activity. From previous work, we know that changes in the molecular structure of the E-cadherin ectodomain at the cell surface are probably involved in allosteric regulation of adhesive binding (Petrova *et al.*, 2012; Shashikanth *et al.*, 2015). These changes are mediated from inside the cell, across the membrane, and out toward the distal homophilic binding regions at the N-termini. In this context, the G239R mutation is particularly interesting because it seems to uncouple adhesive binding activity of the ectodomain from p120-catenin–related signaling activities in the cytoplasm. Therefore it appears to be a mutation that specifically interferes with the transduction of signaling across the membrane, that is, inside-out signaling.

Many regions of the cadherin ectodomain seem to be involved in adhesion activation. Activating mAbs and mAbs that distinguish activity states recognize conformational epitopes at the interfaces between the all of the various EC domains (Petrova *et al.*, 2012). Consistent with this idea, the HDGC and CLP mutations that strongly inhibit adhesion activation are located near the interface between EC2 and EC3, raising the possibility that this interface plays a particularly important role in activation and adhesion regulation. However, the mutations that partially inhibit activation are distributed all over EC1, EC3, EC4, and EC5; perhaps they affect the adhesive conformation or changes indirectly and weakly compared with the interfaces. Of course, it is unlikely that the collection of mutations that affect activation are saturating, since their identification depends on correlations with human disease, nor is it likely that activating and distinguishing mAbs recognize all the important regions. Nonetheless, these mutations provide starting points for elucidating the molecular changes underlying E-cadherin adhesion activation.

There are several ways in which cadherin regulation could be important in cancer. The first is a variation on the standard model in which E-cadherin adhesion is required to keep cells associated with the primary tumor mass and prevent their dissociation, invasion, and migration as individual cells, subsequent dissemination, and metastasis. In this way, a defect in cadherin activation or a reduction in cadherin adhesive activity would play a similar, if subtler, role as the complete loss of E-cadherin expression. Given that complete loss of E-cadherin is often associated with metastasis, this is a compelling model. An alternative is that dynamic regulation of E-cadherin plays a role in mediating collective cell migration that is important for metastasis. Cadherins and collective cell migration have been shown to be important in mammary tumor metastasis (Cheung *et al.*, 2013; Shamir *et al.*, 2014), as well as in normal collective cell invasive movements, such as neural crest migration (Theveneau *et al.*, 2010; Theveneau and Mayor, 2012) and border cell migration in *Drosophila* oogenesis (Geisbrecht and Montell, 2002; Cai *et al.*, 2014). Given the importance of cadherin regulation in morphogenetic cell rearrangement (Brieher and Gumbiner, 1994; Zhong *et al.*, 1999; Gumbiner, 2005), it is likely that spatial and temporal control of adhesive activity can influence collective cell invasion in metastasis.

Similarly, because an E-cadherin mutation associated with cleft lip and palate exhibits impaired adhesion activation, regulation of E-cadherin activity may also be involved in this congenital birth defect, which arises due to the failure of bilateral tissues to properly fuse. Proper fusion involves cell rearrangements and cell convergence movements (Kim *et al.*, 2015), similar to convergence and extension morphogenesis in gastrulating embryos (Keller, 2002), which we previously showed to depend on cell surface cadherin regulation (Brieher and Gumbiner, 1994; Zhong *et al.*, 1999). Thus, whereas basal adhesive activity can keep cells organized in these developing orofacial tissues, regulation may be required for their proper morphogenesis.

There are many other ways in which E-cadherin mutations could affect tumorigenesis besides loss of cell adhesion function. The HDGC mutations that cause constitutive adhesion activation are of particular interest in this regard, since it is not expected that they would promote tumors by mediating too little adhesion. In fact, two recent studies demonstrated that increased adhesion in cell clusters associated with cytokeratin and/or desmosomal protein expression are important intermediates in mammary metastasis (Aceto *et al.*, 2014; Cheung *et al.*, 2016). Further, we cannot yet explain the nature of the deficits caused by those mutations that behave like WT in the CHO cell and colo205 cell adhesion assays. It is possible that some of the mutations affect the stability and/or trafficking of E-cadherin, as occasionally reported for some mutations, especially in the cytoplasmic domain (Suriano *et al.*, 2003; Vogelaar *et al.*, 2013). Such effects were not investigated through our functional assays, which focused on using cells with similar levels of surface expression. However, except for the EED > AAA p120-catenin binding mutation (Maiden *et al.*, 2016), we had no trouble achieving WT levels of surface expression for any of the mutants, and the total levels of protein expression in both CHO and colo205 cell lines appeared roughly similar for all of them (unpublished data). E-cadherin could also affect tumorigenesis by influencing the state of other cell junctions or a variety of signaling pathways, some of which depend on the adhesive activity state of the E-cadherin, such as the Hippo and Wnt signaling pathways and rho-family GTPase signaling (Gumbiner *et al.*, 1988; Gottardi *et al.*, 2001; Noren *et al.*, 2001; McLachlan *et al.*, 2007; Onder *et al.*, 2008; Kim *et al.*, 2011; Gomez *et al.*, 2015). Additional signaling pathways are influenced by E-cadherin interactions with other surface receptors, such as growth factor receptors (Qian *et al.*, 2004; Curto *et al.*, 2007; Perrais *et al.*, 2007; Mateus *et al.*, 2009) and those mediating heterotypic cell interactions (Higgins *et al.*, 1998; Lecuit *et al.*, 1999; Hubert *et al.*, 2005), which may not depend on the homophilic adhesive bond but could be affected by some of the mutations. Additional in-depth studies would be required to understand how these mutations affect the variety of functions mediated by E-cadherin.

E-cadherin could also potentially influence other steps in tumorigenesis besides initial tumor cell dissociation and invasion, such as tumor growth, extravasation from the vasculature, distal seeding and growth, and so on. We did not observe any effects of activating mAbs on the primary tumor growth in our experimental model, but we cannot exclude this for other tumor types or for the effects of the mutations in HDGC. The exact step of tumor growth and metastasis affected by E-cadherin activity or mutations and the mechanism of action could also vary for different models. Indeed, the multiplicity of potential sites and mechanisms of action may explain the important role of E-cadherin in cancer.

## MATERIALS AND METHODS

### Generation of 4T1-hE cell line for metastasis experiment

4T1-Luc2_Puro cells (Kim *et al.*, 2010) were transfected with pcDNA3 plasmid containing WT human E-cadherin by electroporation using Amaxa Nucleofector (Lonza, Basel, Switzerland) according to the manufacturer's instructions. Cells were selected with neomycin and subcloned by limiting dilutions. Human E-cadherin expression level and population homogeneity were verified by Western blot, immunofluorescence staining, and flow cytometry (Supplemental Figure 1).

### Mouse metastasis experiment

4T1-hE cells were injected into the mammary fat pads of BALB/c mice. On day 3, animals started to receive treatments, either "neutral" E-cadherin–specific mAb 46H7 or E-cadherin–activating mAb 19A11, with 15 animals in each group. Antibodies were injected intraperitoneally twice weekly, 5 mg/kg of weight; on average, for each ~20-g mouse, this was 100 µg/mouse/injection. Caliper measurements of primary tumor were done weekly for 3 wk starting at day 7 postinjection. At wk 4, animals were killed, and whole lungs were removed.

DNA samples were purified from whole-lung lysate using a Qiagen (Valencia, CA) kit and further analyzed for luciferase-2 expression by PCR. Quantitative real time (qRT) PCR was performed using an ABI-3000 instrument (Applied Biosystemsm, Waltham, MA). To count metastatic cell number, a calibration curve was created using 4T1-hE cells mixed with lung homogenate. DNA samples corresponding to 10,000 4T1-hE cells were used as a reference for each qRT PCR run. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene was used to control for total cell material.

Because data in both groups did not have a Gaussian distribution according to the Kolmogorov–Smirnov normality test, the Mann–Whitney *U* test was used to confirm statistical difference between groups. Alternatively, data transformed as $\log_{10}$ showed a normal distribution in the Kolmogorov–Smirnov test and were analyzed by Student's *t* test. All statistical analysis was done using GraphPad Prism software, version 5 (GraphPad Software, La Jolla, CA).

Immunohistochemical staining of paraffin sections was performed on a fee-for-service basis by the Experimental Histopathology Core Facility at the Fred Hutchinson Cancer Research Center (Seattle, WA) with antibodies to mouse E-cadherin, mouse vimentin, and Ki67 using standard procedures optimized for these materials by the facility. Evaluation of the staining was performed by Smitha Pillai, a comparative pathologist at the Fred Hutchinson Cancer Research Center,

### Analysis of E-cadherin disease–related mutations

Colo 205 cells with low endogenous E-cadherin expression (colo-hE-shRNA cells) were produced by E-cadherin knockdown by expressing an E-cadherin shRNA using lentiviral infection. For infection, pLKO.1Puro plasmid from Addgene (Cambridge, MA; 18801; Onder *et al.*, 2008) containing 5′-AAGATAGGAGTTCTCTGATGC-3′ small interfering RNA directed against E-cadherin was introduced into HEK293LT cells using Lipofectamine transfection (Thermo Fisher Scientific, Waltham, MA). To produce a viable virus in HEK293LT cells, pMD2.G envelope and psPAX2 packaging plasmids (Didier Trono, Laboratory of Virology and Genetics, École Polytechnique Fédérale de Lausanne; available from Addgene, 12259 and 12260, respectively) were used according to Trono lab protocol (Trono, 2016). Colo 205 cells were infected with lentivirus-containing HEK293LT supernatant by spinoculation at 3000 rpm for 2 h at 33°C and selected with 10 µg/ml puromycin for 5 d. Selected cells were subcloned by limiting dilution, and the clone with the lowest E-cadherin expression was expanded and used in all further experiments.

To address how naturally occurring gastric cancer and CLP-related E-cadherin mutations influence E-cadherin–mediated adhesion, CHO cells (which do not express endogenous cadherins) and Colo 205 cells with low E-cadherin expression (colo-hE-shRNA cells) were infected with lentivirus containing either WT or mutant E-cadherin expression constructs. The WT E-cadherin PCR product was cloned into a pENTR11 Gateway cloning plasmid. Mutations were introduced by site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit; 200521; Agilent, Santa Clara, CA). The WT and mutant E-cadherins were then cloned into pLX304-Blast Gateway lentiviral vector (25890; Addgene) and used for lentivirus production in HEK293LT cells together with pMD2.G envelope and psPAX2 packaging plasmids (Trono, 2016). CHO cells and colo-hE-shRNA cells were infected

by spinoculation as described and selected with 10 µg/ml blasticidin for 10 d. Mutant and WT E-cadherin expression level and population homogeneity were verified by flow cytometry at the University of Virginia Flow Cytometry Facility (Supplemental Figures 2 and 3). To achieve homogeneity and expression compared with those in parental Colo205 mutant E-cadherin, live-cell populations were stained with HECD-1 mAb and sorted by a Vantage instrument. Flow cytometry–based E-cadherin expression level verification in CHO and Colo205 cells was performed just before every experiment using a desktop MoxiFlow device (ORFLO Technologies, Ketchum, ID).

### Flow cytometry

For flow cytometry analysis, adherent cells were lifted by 0.01% trypsin in phosphate-buffered saline containing 2 mM $CaCl_2$ to prevent cadherin digestion. Low-adherent Colo205 cells were collected by pipetting. Cell suspension was filtered through cell strainer, and single cells were stained using human E-cadherin–specific mAbs 19A11, 27D2, and 46H7 (Petrova *et al.*, 2012) or HECD1 (Shimoyama *et al.*, 1989) as primary and goat anti-mouse labeled with Alexa 546 (Biolegend, San Diego, CA) as secondary antibodies. E-cadherin expression level was determined by FACSCalibur (BD Bioscience, San Jose, CA) or MoxiFlow instruments and analyzed by FlowJo software (Ashland, OR).

### Laminar flow cell adhesion assay

The laminar flow cell adhesion assay was conducted as described previously (Yap *et al.*, 1997; Chappuis-Flament *et al.*, 2001). In brief, the cells were trypsinized in the presence of 2 mM calcium, allowed to attach to glass capillary tubes coated with E-cadherin for 10 min, and washed away for 30 s at an indicated flow rate. The cells remaining after the wash were counted, and the adhesion percentage was calculated.

### Colo205 activation assay

Cells were seeded in 24-well plates, cultured overnight, and treated with control or adhesion-activating substances for the indicated time, typically 5 h or overnight for 1 µg/ml mAb Fab-fragments or 7 nM staurosporine or 0.001% trypsin as described previously (Aono *et al.*, 1999; Petrova *et al.*, 2012). Alternatively they were activated by treatment for 3 h with 60 mM LiCl or for 1 h with 10 µM nocodazole (Maiden *et al.*, 2016). The extent of cell aggregation and compaction was documented by photographing numbers fields using a bright-field microscope. For the quantitative assay of activation, cells were lifted by pipetting and filtered through a cell strainer to remove cell aggregates, and the single cells were counted using a MoxiFlow instrument. The loss of single cells after a set time is a measure of their participation in cell aggregation and reflects the adhesive abilities of E-cadherin mutants.

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