Cdc42 Regulates E-cadherin Ubiquitination and Degradation through an Epidermal Growth Factor Receptor to Src-mediated Pathway*

Yi Shen¹, Dianne S. Hirsch¹, Christy A. Sasiela², and Wen Jin Wu³

From the Division of Monoclonal Antibodies, Office of Biotechnology Products, Office of Pharmaceutical Science, Center for Drug Evaluation and Research, United States Food and Drug Administration, Bethesda, Maryland 20892

E-cadherins play an essential role in maintaining epithelial polarity by forming Ca²⁺-dependent adherens junctions between epithelial cells. Here, we report that Ca²⁺ depletion induces E-cadherin ubiquitination and lysosomal degradation and that Cdc42 plays an important role in regulating this process. We demonstrate that Ca²⁺ depletion induces activation of Cdc42. This in turn up-regulates epidermal growth factor receptor (EGFR) signaling to mediate Src activation, leading to E-cadherin ubiquitination and lysosomal degradation. Silencing Cdc42 blocks activation of EGFR and Src induced by Ca²⁺ depletion, resulting in a reduction in E-cadherin degradation. The role of Cdc42 in regulating E-cadherin ubiquitination and degradation is underscored by the fact that constitutively active Cdc42(F28L) increases the activity of EGFR and Src and significantly enhances E-cadherin ubiquitination and lysosomal degradation. Furthermore, we found that GTP-dependent binding of Cdc42 to E-cadherin is critical for Cdc42 to induce the dissolution of adherens junctions. Our data support a model that activation of Cdc42 contributes to mesenchymal-like phenotype by targeting of E-cadherin for lysosomal degradation.

Adherens junctions are one of the major structures responsible for cell-cell adhesion between epithelial cells, and their formation is required for the establishment of a differentiated epithelium (1). Adherens junctions form by Ca²⁺-dependent homophilic interactions between neighboring cells mediated by type I receptors known as cadherins (2, 3). Adherens junctions are dynamic structures that are remodeled as needed based on cellular conditions such as during embryonic development and wound healing. Remodeling of adherens junctions requires endocytosis and recycling of E-cadherin (4). During epithelial tumor progression, disruption of adherens junctions and decreased E-cadherin expression occurs and contributes to epithelial to mesenchymal transitions (EMT)⁴ (5). Although sustained repression of E-cadherin occurs at the transcriptional level, initial decreases in E-cadherin levels likely occur by lysosomal degradation (6, 7). Mechanisms regulating the trafficking of E-cadherin for lysosomal degradation are poorly understood.

Overexpression of the epidermal growth factor receptor (EGFR) correlates with metastasis in breast cancer and other carcinomas. EGF stimulation of epithelial cells has been shown to disrupt adherens junctions (8), and chronic stimulation causes up-regulation of E-cadherin transcriptional repressors such as TWIST and Snail (9, 10). Conversely, inhibition of EGFR signaling contributes to increased E-cadherin protein levels and restoration of adherens junctions in lung cancer and breast cancer cell lines (11, 12). Although it is clear that increased EGFR signaling contributes to sustained repression of E-cadherin expression, it is not known whether or not EGFR contributes to the initial down-regulation of E-cadherin by lysosomal degradation.

Src, a non-receptor tyrosine kinase, is a central regulator of signaling downstream of EGFR and has been shown to regulate EMT by disrupting adherens junctions (13, 14). Mechanically, Src alters E-cadherin trafficking by redirecting E-cadherin from a recycling pathway to a lysosomal-targeting pathway (7). In Madin-Darby canine kidney cells Src phosphorylation of E-cadherin leads to binding of the E3 ligase Hakai; Hakai then ubiquitinates E-cadherin, resulting in E-cadherin endocytosis (15). The downstream effect of Hakai-mediated E-cadherin ubiquitination and endocytosis has not been characterized.

Rho family GTPases, including Cdc42, Rac1, and RhoA, have all been found to regulate adherens junctions (16, 17). The majority of data indicate that Rho GTPases contribute to the establishment and stabilization of adherens junctions in mouse L fibroblasts expressing E-cadherin (EL cells) (18, 19). For instance, Cdc42 and Rac1 stabilize adherens junctions in Madin-Darby canine kidney cells, and activation of both contributes to the initial formation of adherens junctions (20–22). It has been recently reported that FRG, a Cdc42-specific guanine nucleotide exchange factor, regulates cell junction assembly through activation of Cdc42 in Madin-Darby canine kidney cells (23). However, there are also data that suggest Rac1 and Cdc42 contribute to dissolution of adherens junctions in human epidermal keratinocytes (24, 25).

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¹ Both authors contributed equally to this work.
² An Interagency Oncology Task Force Fellow supported by the United States Food and Drug Administration and the NCI, National Institutes of Health, Bethesda, MD.
³ To whom correspondence should be addressed: Division of Monoclonal Antibodies, OB/P/OPS/CDE/FDA, Bldg. 29B, Rm. 3NN-15, 29 Lincoln Dr., Bethesda, MD 20892-4555. Tel.: 301-827-0253; Fax: 301-827-0852; E-mail: wen.wu@fda.hhs.gov.

The abbreviations used are: EMT, epithelial to mesenchymal transitions; EGFR, epidermal growth factor (EGF) receptor; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; siRNA, short interfering RNA; WCL, whole cell lysates; PBD, p21 binding domain; GST, glutathione S-transferase; wt, wild type; ERK, extracellular signal-regulated kinase.
of adherens junctions is in part mediated by stimulating endocytosis of E-cadherin (25). Overexpression of constitutively active Cdc42 was also found to prevent adherens junction formation in keratinocytes, suggesting that Cdc42 may negatively regulate adherens junction establishment (26).

Activation of Cdc42 in fibroblast cells has been reported to inhibit Cbl-mediated ubiquitination and degradation of EGFR, resulting in cellular transformation due to the accumulation of EGFR and sustained EGF-stimulated ERK activation (27). In MDA-MB-231 breast cancer cells, down-regulation of Cdc42 restores EGFR degradation and decreases cell migration (28). Based on this information, we hypothesized that Cdc42 might regulate E-cadherin degradation through an ubiquitin-mediated pathway to dissolve adherens junctions and positively regulate cell migration. Given the roles of Cdc42, Src, and EGFR in adherens junction dynamics, we addressed the question of whether signaling through Cdc42, EGFR, and Src cooperatively contributes to the dissolution of adherens junctions, leading to E-cadherin ubiquitination and degradation.

Here, we provide evidence that after endocytosis induced by Ca\(^{2+}\) depletion, E-cadherin is ubiquitinated and targeted for lysosomal degradation and that Cdc42 plays a critical role in regulating this process through an EGFR to Src signaling pathway. We demonstrate that Cdc42 is activated under Ca\(^{2+}\)-free conditions. Activation of Cdc42 subsequently initiates the activation of EGFR and Src. Activated Src in turn tyrosine-phosphorylates E-cadherin, leading to Hakai-mediated E-cadherin ubiquitination. These data are supported by the results that silencing Cdc42 inhibits activation of EGFR and Src, resulting in a reduction in E-cadherin degradation. When Cdc42 is constitutively activated, as is the case for Cdc42(F28L), an oncogenic mutant that undergoes spontaneous activation (29, 30), the persistent activation of EGFR and Src leads to a significant enhancement in E-cadherin ubiquitination and degradation, causing the dissolution of E-cadherin-based adherens junctions and an increased rate of cell migration. Furthermore, our data indicate that activated Cdc42 binds to E-cadherin in a GTP-dependent manner, and the binding of Cdc42 is essential for Cdc42 to induce the dissolution of adherens junctions.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies against E-cadherin (clone 36), EGFR, and activated EGFR were from BD Transduction Laboratories. Antibodies against the N terminus of E-cadherin (clone HEC12) and against v-Src (mouse monoclonal antibody 327) were from Calbiochem. Anti-HA antibody was from Covance (Richmond, CA). Rabbit anti-Src (36D10) was from Cell Signaling (Beverly, MA). Anti-Hakai and anti-ubiquitin antibodies were from Zyomed Laboratories Inc. (South San Francisco, CA). Anti-phosphotyrosine (clone 4G10) antibody was from Upstate Biotechnology, Inc., (Lake Placid, NY). Anti-LAMP1 (H4A3) antibody, developed by J. Thomas August and James E. K. Hildreth, was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Alexa Fluor-conjugated secondary antibodies were from Invitrogen Molecular Probes (Eugene, OR).

**Cell Culture and Transient and Stable Transfections**—MCF-7 cells, from American Type Culture Collection (Manas-sas, VA), were grown in DMEM supplemented with 10% fetal bovine sera (FBS) and antibiotic/antimycotic. Cell culture media, trypsin, Lipofectin transfection agent, and PLUS reagent were from Invitrogen. Cells stably expressing Cdc42(F28L) were generated using methods described previously (29, 30). Transient transfections using Lipofectin and PLUS reagent were done according to the manufacturer’s protocol. SMARTpool short interfering RNA (siRNA) targeting human Cdc42, human Hakai, and control non-targeting siRNA were purchased from Dharmacon (Chicago, IL). MG132 proteasome inhibitor was purchased from Calbiochem. Tyrphostin AG1478 was purchased from Sigma-Aldrich. For EGFR inhibition studies, cells were seeded in the morning and allowed to attach and spread for 8 h. Me\(_2\)SO (control) or AG1478 (at 10 \(\mu\)M) was then added overnight. Cells were then switched to Ca\(^{2+}\)-free DMEM in the presence of Me\(_2\)SO or AG1478 (10 \(\mu\)M) for the indicated times.

**E-cadherin Ubiquitination and Degradation**—MCF-7 cells were seeded overnight in 10% FBS/DMEM, and then cells were rinsed twice with phosphate-buffered saline before switching to Ca\(^{2+}\)-free DMEM. At the indicated times cells were harvested, and whole cell lysates (WCL) were subjected to immunoprecipitation using an anti-E-cadherin antibody. Ubiquitinated E-cadherin was detected by Western blot with anti-ubiquitin antibody. The immunoprecipitated E-cadherin as indicated above or protein levels of E-cadherin in WCL harvested at the indicated time points were detected by Western blot using anti-E-cadherin antibody for assessing E-cadherin degradation.

**E-cadherin Internalization and Recycling Assays**—Internalization and recycling assays were done as previously described (4, 31). MCF-7 cells and Cdc42(F28L)-expressing MCF-7 (clone 19) cells were seeded overnight at 1.5 \(\times\) 10\(^6\) cells/35-mm dish in complete media. Cells were biotinylated using EZ-Link Sulfo-NHS-SS-Biotin according to the manufacturer’s protocol (Pierce). For recycling assays, accumulation of biotinylated proteins in early or sorting endocytic compartments was accomplished by incubating cells for 2 h at 18 °C (32–34). Cell surface biotinylated proteins were then stripped as previously described (35), and cells were incubated at 37 °C for the indicated times. Protein concentrations were determined using Bradford reagent (Bio-Rad). Biotinylated proteins from radioimmunoprecipitation assay buffer-extracted WCL, and media were affinity-precipitated using immobilized NeutrAvidin\(^\text{TM}\) protein per the manufacturer’s protocol (Pierce). Levels of biotinylated E-cadherin were determined by Western blot using anti-E-cadherin (HECD) antibody.

**p21 Binding Domain (PBD) Assay**—The determination of the levels of GTP-bound Cdc42 were done using the GST-PBD of p21-activated kinase (PAK) as described previously (36).

**Immunofluorescence**—Cells were seeded overnight on chamber slides at 1 \(\times\) 10\(^5\) cells/well in 10% FBS/DMEM. Cells were then rinsed twice with phosphate-buffered saline and incubated for the indicated times in DMEM or Ca\(^{2+}\)-free DMEM. Cells were fixed and stained as previously described (28). Actin was detected using rhodamine-conjugated phalloidin (Invitrogen Molecular Probes). Src was detected using rabbit anti-Src (36D10).
Migration Assays—Boyden chamber migration assays, fixation, and staining were done as described previously (37). Cells were incubated for 5 h at 37 °C with 5% CO₂ and then fixed and stained. Wound healing assays are described in the Fig. 2E legend.

RESULTS
Ca²⁺ Depletion Induces Ubiquitination and Degradation of E-cadherin—Although E-cadherin endocytosis represents an important cellular process that regulates the assembly and disassembly of adherens junction, E-cadherin trafficking after endocytosis is not yet thoroughly understood. We used MCF-7 breast cancer cells, which express high levels of endogenous E-cadherin, as a model system to explore the molecular mechanisms of E-cadherin trafficking.

We set out to investigate whether endocytosed E-cadherin induced by Ca²⁺ depletion can be targeted for lysosomal degradation. Cells were switched to Ca²⁺-free media to initiate E-cadherin endocytosis and harvested at the indicated time points, and then E-cadherin proteins were immunoprecipitated from WCL. Western blotting was used to detect E-cadherin protein levels (Fig. 1A). A significant reduction in E-cadherin protein levels was observed after a 3-h incubation in Ca²⁺-free media. Two lower molecular weight protein bands that are likely E-cadherin degradation products were also recognized by the anti-E-cadherin antibody after incubation of cells in Ca²⁺-free media (Fig. 1A, arrows).

We next tested whether E-cadherin was ubiquitinated upon Ca²⁺ depletion. Significant ubiquitination of immunoprecipitated E-cadherin was observed after a 3-h Ca²⁺ depletion (Fig. 1B, compare lanes 1 and 2, upper image), and E-cadherin ubiquitination was not detected when control IgG was used for immunoprecipitation (lanes 3 and 4). Decreased total protein levels of E-cadherin and the two lower protein bands were also detected in WCL upon Ca²⁺ depletion (lanes 2 and 4 in Fig. 1B, middle image). To confirm the ubiquitination of E-cadherin under Ca²⁺-free conditions, we transiently expressed HA-ubiquitin in MCF-7 cells. The experimental procedures for Fig. 1C were essentially the same as described in Fig. 1B, except the immunoprecipitated E-cadherin was detected by anti-HA antibody. As shown in Fig. 1C, Ca²⁺ depletion significantly induced E-cadherin ubiquitination that was not detected when control IgG was used for immunoprecipitation. To elucidate the role of the proteasome in Ca²⁺ depletion-induced E-cadherin ubiquitination and degradation, we treated cells with MG132, a proteasomal inhibitor. Consistent with the results shown in Fig. 1, B and C, upon Ca²⁺ depletion, E-cadherin was ubiquitinated and degraded (Fig. 1D, lane 3, top three images). However, treatment of cells with MG132 did not prevent E-cadherin degradation but appeared to increase its ubiquitination and degradation (lane 4 in Fig. 1D, top three images), suggesting that Ca²⁺ depletion induced E-cadherin degradation may occur in lysosomes.

We next determined if E-cadherin was transported to lysosomes. As shown in Fig. 1E, E-cadherin colocalized with the lysosomal marker LAMP-1 in a perinuclear compartment after a 1-h Ca²⁺ depletion, whereas there was no significant colocalization between E-cadherin and LAMP-1 when cells were cultured in Ca²⁺-containing media. After 1 h of incubation in Ca²⁺-free media, ~20% of cells had E-cadherin colocalization with LAMP-1 as compared with 5% of cells grown in Ca²⁺ containing media (Fig. 1E, graph). Taken together, we conclude that E-cadherin ubiquitination can be induced by Ca²⁺ depletion, and ubiquitinated E-cadherin is targeted for lysosomal degradation.

![Figure 1. E-cadherin is ubiquitinated and degraded after the switch to Ca²⁺-free media.](Image)
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**FIGURE 2. Disengagement of cell-cell adhesions by Ca\(^{2+}\) depletion activates Cdc42 and enhances cell migration.** A, MCF-7 breast cancer cells were seeded overnight in 10% FBS/DMEM and then switched to DMEM with or without Ca\(^{2+}\) for the indicated times. Arrows, cellular protrusions. Magnification, 200×. B, MCF-7 cells were incubated for 3 h in DMEM with or without Ca\(^{2+}\). Cells were then fixed, permeabilized, and stained with rhodamine-conjugated phalloidin to detect F-actin. Magnification, 2400×. C, MCF-7 cells were incubated in Ca\(^{2+}\)-free media for the indicated times. Activated Cdc42 was affinity-precipitated from WCL by incubating with GST-PBD-agarose. Bound Cdc42 was detected by Western blot for Cdc42. D, migration of MCF-7 cells in 0.1% BSA in Ca\(^{2+}\)-containing or Ca\(^{2+}\)-free DMEM was determined using a Boyden chamber migration assay. 12 µg/ml fibronectin was used as the chemoattractant in the lower chamber. Upper and lower chambers were separated by an 8.0 µm polycarbonate membrane. 5 h after seeding cells were fixed in methanol and then Giemsa-stained. Data are the mean ± S.D. of three independent experiments. E, wound healing assays were done on MCF-7 cells seeded overnight in DMEM medium supplemented with 10% FBS in 12 well plates. Cells were then scratch-wounded across the cell monolayer using a 1000-µl micropipette and grown in DMEM with or without Ca\(^{2+}\). Phase contrast images were taken at the indicated times. Magnification, 200×.

**Ca\(^{2+}\) Depletion Triggers the Activation of Cdc42 and Enhances the Rate of Cell Migration—**Under Ca\(^{2+}\)-free conditions, we observed that MCF-7 cells gradually dissociated, depolarized, and formed numerous cellular protrusions (Fig. 2A, right panels). Results from staining cells with rhodamine-conjugated phalloidin to detect actin suggested that the cellular protrusions observed under Ca\(^{2+}\)-free conditions were microspikes or filopodia (Fig. 2B). It is well established that activation of Cdc42 triggers the formation of filopodia (38). We then asked whether Ca\(^{2+}\) depletion induced the activation of Cdc42. A GST-PBD pulldown assay provided evidence that levels of activated Cdc42 were elevated under Ca\(^{2+}\)-free conditions (Fig. 2C, compare 0 h to 1 and 3 h). Taken together, these data suggest that the Ca\(^{2+}\)-free media-induced filopodium formation might be the direct consequence of Cdc42 activation. Activation of Cdc42 has been implicated in the regulation of cell migration (38). We next tested whether depletion of Ca\(^{2+}\) could increase the rate of cell migration. As measured by Boyden chamber and wound healing migration assays, cell migration in Ca\(^{2+}\)-free media was significantly increased as compared with that in Ca\(^{2+}\)-containing media (Fig. 2, D and E).

**Activation of Cdc42 Enhances Ubiquitination and Degradation of E-cadherin, Resulting in Dissolution of Adherens Junctions and an Increased Rate of Cell Migration—**We next asked whether activation of Cdc42 had the ability to regulate E-cadherin ubiquitination and degradation. MCF-7 cell lines stably expressing different levels of Cdc42(F28L) (clones 19, 13, and 7; see Fig. 3B) and vector control cells were incubated in DMEM without Ca\(^{2+}\) for the indicated times, and then E-cadherin protein levels in WCL were detected by Western blot. As shown in Fig. 3A, increased reduction in E-cadherin protein levels was observed in cells stably expressing Cdc42(F28L) (clones 19, 13, and 7). However, the extent of E-cadherin degradation under Ca\(^{2+}\)-free conditions was significantly increased in cells with a high expression of Cdc42(F28L) (clones 19 and 13) as compared with that in vector control cells such that the total E-cadherin protein levels were dramatically reduced in clones 19 and 13 cells after cells were incubated in Ca\(^{2+}\)-free media for 1 h (Fig. 3A, lanes 3–5 to lane 2). The extent of E-cadherin ubiquitination was not distinguishable among the different clones of Cdc42(F28L)-expressing MCF-7 cells. This may be due to the detection limit of this particular assay. Taken together, these data indicate that activation of Cdc42 enhanced E-cadherin degradation under Ca\(^{2+}\)-free conditions.

We next tested whether activation of Cdc42 led to the dissolution of E-cadherin-based adherens junctions. Although transient expression of wild-type Cdc42 and dominant negative Cdc42(T17N) had no effect on E-cadherin localization at adherens junctions (Fig. 3D, top and bottom panels), expression of Cdc42(Q61L) produced filopodium formation and induced the disappearance of E-cadherin-based adherens junctions (Fig. 3D, middle panels). Adherens junctions were then examined in the MCF-7 stables cell lines. MCF-7 vector control cells had robust colocalization between E-cadherin and actin at adherens junctions (Fig. 3E, left panels). In contrast, filopodia induced by the stable expression of Cdc42(F28L) interrupted E-cadherin-based adherens junctions (Fig. 3E, right panels). Taken together, these data indicate that expression of activated Cdc42 in MCF-7 cells disrupts the polarized epithelial morphology and contributes to the development of a mesenchyme-like morphology.

A strong correlation between loss of E-cadherin at the level of the cell surface and enhanced cell invasiveness has been described (39). We tested whether Cdc42(F28L) had the ability...
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Given that Cdc42 is an important regulator of cell migration, we hypothesized that activation of Cdc42 may influence E-cadherin endocytosis and recycling. To test this hypothesis, we used a biotinylation endocytosis/recycling assay system previously described (4). We found that biotinylated E-cadherin was internalized to a similar extent for both vector control cells and Cdc42(F28L)-expressing cells at 15 min after shifting the temperature to 37 °C (Fig. 4A, top panel, compare lanes 2 and 6), indicating that active Cdc42(F28L) did not affect the rate of E-cadherin internalization. Although the protein levels of internalized E-cadherin were slightly reduced in vector control cells over a time period of 15 min to 1 h (compare lanes 2–4 in the top panels), internalized E-cadherin levels were significantly decreased in Cdc42(F28L)-expressing cells (Fig. 4A, compare lanes 6 and 8). These data indicate that the inter-
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Tumors are characterized by uncontrolled growth. The ability of tumor cells to acquire this property is related to the ability to change their morphology by dynamic cell motility, migration, and invasiveness. Cdc42, a member of the Ras superfamily, regulates the subcellular organization of the actin cytoskeleton, focal adhesions, and microfilament dynamics, which are important for cell migration and adhesion. Here, we examined whether Cdc42 regulates E-cadherin levels, which are implicated in the regulation of cell motility and adhesion. We show that Cdc42 regulates E-cadherin levels at the cell surface and that this regulation is mediated by E-cadherin ubiquitination and degradation. Our results indicate that Cdc42 regulates E-cadherin levels in a manner that affects cell motility and adhesion. This finding suggests that Cdc42 plays a role in the regulation of E-cadherin levels, which may have implications for the development of new therapeutic strategies for the treatment of cancer.

Figure 5. Ca²⁺ depletion induces activation of Src, and Cdc42(F28L) increases Src activity in MCF-7 cells. (A) MCF-7 cells were grown in Ca²⁺-free DMEM media for the indicated times. WCL were subjected to either anti-phosphotyrosine antibody (clone 4G10) or anti-Src-Tyr(P)-416 antibody. Actin (clone 4G10) and Src were detected by Western blot analysis. (B) MCF-7 vector control cells and MCF-7 Cdc42(F28L) clone 19 cells were grown as described in A. WCL were subjected to anti-phosphotyrosine (clone 4G10) and anti-Src-Tyr(P)-416 immunoprecipitations. Bound proteins were resolved by SDS-PAGE followed by Western blot analysis using antibodies against either Src or E-cadherin. (C) MCF-7 vector control cells were grown as described in A. WCL were subjected to anti-phosphotyrosine (clone 4G10) and anti-Src-Tyr(P)-416 immunoprecipitations. Bound proteins were resolved by SDS-PAGE followed by Western blot analysis using antibodies against either Src or E-cadherin. (D) MCF-7 vector control cells were grown as described in A. WCL were subjected to anti-phosphotyrosine (clone 4G10) and anti-Src-Tyr(P)-416 immunoprecipitations. Bound proteins were resolved by SDS-PAGE followed by Western blot analysis using antibodies against either Src or E-cadherin.
Ca\(^{2+}\) depletion. As shown in Fig. 7A, tyrosine phosphorylation of EGFR was enhanced upon Ca\(^{2+}\) depletion for 15 min, indicating that the activity of EGFR is enhanced (top panel). These data are consistent with another report (40). Cdc42 activation levels also increased after a 15-min incubation in Ca\(^{2+}\)-free DMEM media (Fig. 7A, bottom panel). We next compared activation levels of the EGFR downstream effector ERK between vector control MCF-7 cells and Cdc42(F28L) #19 cells. When
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grown in Ca\(^{2+}\)-containing DMEM media, MCF-7 Cdc42(F28L)-expressing cells had increased levels of activated ERK as compared with MCF-7 vector control cells (Fig. 7A, middle panels). EGFR protein levels were also increased in MCF-7 Cdc42(F28L) cells as compared with vector control (data not shown). Upon Ca\(^{2+}\) depletion, cells stably expressing Cdc42(F28L) differed from vector control cells in that they did not have an increase in ERK activation levels, indicating that ERK was constitutively active. MCF7 Cdc42(F28L) clones 7 and 13 also had increased activated ERK as compared with vector control cells (Fig. 7B). We next determined if ERK was activated in MCF7 cells after EGF treatment. After overnight serum starvation, MCF-7 cells were stimulated with EGF (100 ng ml\(^{-1}\)) for 15 min; ERK1 and ERK2 were activated after EGF treatment (Fig. 7C). These data indicate that Ca\(^{2+}\) depletion sufficiently activates EGFR coupled signaling in MCF-7 cells.

We next assessed whether or not EGFR regulated E-cadherin degradation. We first determined if EGFR treatment affected E-cadherin degradation. MCF-7 cells were incubated in Ca\(^{2+}\)-free DMEM for the indicated times in the absence or presence of EGF (100 ng ml\(^{-1}\)). Increased E-cadherin degradation occurred when cells were incubated in the presence of EGF (Fig. 7D, compare lanes 2 and 3 to lanes 5 and 6). As expected, after EGF treatment, EGFR protein levels decreased. The same was true when MDA-MD-468 breast cancer cells, which express high levels of endogenous EGFR, were stimulated by EGF (data not shown). We next tested whether or not inhibiting EGFR activation using the EGFR inhibitor AG1478 decreased E-cadherin degradation after Ca\(^{2+}\) depletion. As shown in Fig. 7E, AG1478 was able to inhibit Ca\(^{2+}\) depletion-induced E-cadherin degradation in Cdc42(F28L)-expressing cells. Similar results were obtained in vector control cells (data not shown).

We then tested whether Cdc42 was necessary for the activation of EGFR and Src upon Ca\(^{2+}\) depletion. After transfection with either control non-targeting siRNA or Cdc42-targeting siRNA, tyrosine-phosphorylated EGFR and Src were immunoprecipitated using clone 4G10 anti-phosphotyrosine antibody. Activated EGFR and activated Src were then detected by Western blot (Fig. 7F). In cells transfected with control non-targeting siRNA, the levels of tyrosine-phosphorylated EGFR and Src were enhanced upon Ca\(^{2+}\) depletion (Fig. 7F, lanes 1 and 2, top two panels). In contrast, silencing Cdc42 blocked the activation of EGFR and Src induced by Ca\(^{2+}\) depletion (Fig. 7F, lanes 3 and 4, top two panels). Untransfected cells were subjected to an anti-HA immunoprecipitation to control for nonspecific protein precipitation.

We next determined if reducing Cdc42 protein levels affected E-cadherin degradation. MCF-7 cells were transfected with either control non-targeting siRNA or Cdc42-targeting siRNA. After Ca\(^{2+}\) depletion for 3 h, levels of E-cadherin were determined by E-cadherin Western blot of immunoprecipitated E-cadherin and of E-cadherin in WCL. After 3 h of incubation in Ca\(^{2+}\)-free media, the level of E-cadherin in cells transfected with control siRNA was significantly decreased (Fig. 7G, compare lanes 1 and 2). However, silencing Cdc42 inhibited E-cadherin degradation induced by Ca\(^{2+}\) depletion such that the level of E-cadherin in cells transfected with Cdc42 siRNA was about 3-fold higher than that in cells transfected with control siRNA (Fig. 7G, compare lane 4 to lane 2), suggesting that Cdc42 activity is required for E-cadherin degradation induced by Ca\(^{2+}\) depletion.

Cdc42 Binds to E-cadherin and Hakai and Regulates Intracellular Localization of Hakai Proteins—The involvement of Cdc42 in the regulation of E-cadherin-based adherens junctions raised the possibility that Cdc42 might interact with E-cadherin. MCF-7 cells were transiently transfected with HA-tagged Cdc42 expression vectors, and then WCL were subjected to anti-HA immunoprecipitations. E-cadherin preferentially bound to constitutively activated Cdc42(Q61L) and Cdc42(F28L) (Fig. 8A, top panel). IQGAP1, which is a target protein of Cdc42 (41), was also found in the complex of activated Cdc42 and E-cadherin (data not shown). E-cadherin also associated with Cdc42(F28L) that was stably expressed in MCF-7 cells (Clone 19) (Fig. 8B). In GST pulldown assays, endogenous E-cadherin expressed in MCF-7 cells bound preferentially to constitutively activated Cdc42 (Cdc42(Q61L)) but not GST control or dominant negative Cdc42 (Cdc42(T17N)) (Fig. 8C). Taken together, these data indicate that Cdc42 interacts with E-cadherin in a GTP-dependent manner similar to the interaction between Cdc42 and its well-established target protein, IQGAP.

Hakai has been reported to associate with E-cadherin and function as an E3 ligase that ubiquitinates E-cadherin (15). We first asked whether Hakai was responsible for the E-cadherin ubiquitination observed under Ca\(^{2+}\)-free conditions. After a 48-h transfection with either control non-targeting siRNA (Fig. 8D, lanes 1, 2, 5, and 6) or Hakai-targeting siRNA (lanes 3, 4, 7, and 8), cells were switched to DMEM with or without Ca\(^{2+}\) for 3 h. Endogenous E-cadherin was immunoprecipitated using an anti-E-cadherin antibody, and the levels of ubiquitinated E-cadherin were assessed by Western blot. Silencing Hakai significantly reduced levels of E-cadherin ubiquitination induced by Ca\(^{2+}\) depletion in vector control cells as well as in Cdc42(F28L)-expressing cells (compare lane 2 to lane 4 and lane 6 to lane 8, respectively). The basal level of E-cadherin ubiquitination, as shown in Fig. 8D, in cells stably expressing Cdc42(F28L) was slightly elevated as compared with that in vector control cells (compare lanes 5 to 1). However, the levels of E-cadherin ubiquitination in Cdc42(F28L)-expressing cells was significantly higher than that in vector control cells upon Ca\(^{2+}\) depletion (compare lanes 6 to 2). Data in Fig. 8D also provide additional evidence that activated Cdc42 enhanced E-cadherin ubiquitination. The ability of Cdc42 to bind E-cadherin and to regulate E-cadherin ubiquitination and degradation led to the question of whether there was any functional linkage between Cdc42 and Hakai in cells. As shown in Fig. 8E, Hakai was found in a protein complex that contained activated Cdc42 and E-cadherin.

It has been reported that Hakai binds to E-cadherin that has been phosphorylated by Src and that after Hakai binding, E-cadherin is ubiquitinated (15). Given Src is activated upon Ca\(^{2+}\) depletion, we asked whether Ca\(^{2+}\) depletion affected E-cadherin tyrosine phosphorylation and whether or not Ca\(^{2+}\) depletion induced E-cadherin binding to Src or Hakai. After Ca\(^{2+}\) depletion, E-cadherin was tyrosine-phosphorylated (Fig. 8F, top panel). Additionally, E-cadherin, Hakai,
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We examined whether Cdc42 had the ability to regulate the intracellular localization of Hakai. In MCF-7 cells, Hakai localized at punctate structures throughout the cytoplasm and was not detected at cell-cell junctions (Fig. 8G). Overexpression of Cdc42(T17N) caused no changes in the intracellular localization of Hakai in MCF-7 cells, whereas in cells expressing Cdc42(Q61L) or Cdc42(F28L), Hakai translocated to a perinuclear compartment (Fig. 8G). Ca\(^{2+}\) depletion also induced Hakai translocation from the cytoplasm to a perinuclear compartment where it colocalized with E-cadherin (Fig. 8H). Overall, these data, when taken together with data shown in Fig. 3, suggest that E-cadherin may function as a downstream target of Cdc42, and binding of activated Cdc42 to E-cadherin is essential for the dissolution of E-cadherin-based adherens junctions.

Here, we describe a new role for Cdc42 in the regulation of E-cadherin-based adherens junctions. Based on a number of lines of evidence provided in this study, we propose a model for Cdc42-mediated regulation of E-cadherin ubiquitination and degradation (Fig. 9). In this model, Ca\(^{2+}\) depletion disengages homophilic interactions between E-cadherin and activates Cdc42. Activated Cdc42 then enhances EGFR signaling to mediate activation of Src. This leads to tyrosine phosphorylation of E-cadherin, which allows Hakai to bind to and subsequently ubiquitinate E-cadherin, thus resulting in E-cadherin lysosomal degradation. In cells overexpressing constitutively active Cdc42, the signaling through EGFR to Src is up-regulated, causing the enhancement in Hakai-mediated E-cadherin ubiquitination and targeting of E-cadherin for lysosomal degradation.
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**DISCUSSION**

Cdc42 Regulates E-cadherin Ubiquitination and Degradation—During EMTs, E-cadherin-dependent adhesion is down-regulated, and cells lose their adhesive contact enabling cell migration (14, 42). Here, we show that loss of a polarized epithelial morphology and the acquisition of mesenchyme-like phenotype, which is accompanied by E-cadherin ubiquitination and lysosomal degradation, can be induced by Ca$^{2+}$ depletion in MCF-7 cells. This indicates that a Ca$^{2+}$ switching technique is a suitable method for studying signal transduction during the early stages of E-cadherin down-regulation and determining its biological relevance. Moreover, we demonstrate that overexpression of active Cdc42, Src, or EGFR causes morphological changes similar to that induced by Ca$^{2+}$ depletion (Figs. 3 and 6) and that the rate of cell migration was increased in MCF-7 cells expressing Cdc42(F28L) as compared with vector control cells.

Data presented here extend our understanding of Src-mediated E-cadherin degradation. In Madin-Darby canine kidney cells, v-Src expression regulates ubiquitin-dependent E-cadherin lysosomal degradation (7). However, upstream regulation of Src was not elucidated in these studies. Our data indicate that Src signals downstream of Cdc42 and EGFR to mediate E-cadherin ubiquitination and degradation. Stable expression of Cdc42(F28L) leads to constitutive activation of Src in MCF-7 cells. Silencing Cdc42 prevents Src activation induced by Ca$^{2+}$ depletion and results in a decreased rate of E-cadherin degradation.

Several lines of evidence suggest functional connections between EGFR and E-cadherin (43). For instance, chronic EGFR stimulation of epidermoid carcinoma cells initiates down-regulation of E-cadherin (10). Lowy and co-workers (40) reported that E-cadherin was found in a protein complex with EGFR, and Ca$^{2+}$ depletion enhanced EGF-stimulated tyrosine phospho-
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Implication for a Role of Cdc42 in Cancer Progression—E-cadherin has been extensively studied, and loss of function has been associated with cancer progression, invasion, and metastasis (48–50). Cdc42 has been implicated in regulation of cell migration, invasiveness, and metastasis (51). Overexpression of Cdc42 has been found in human breast cancers, and it is believed that the elevated expression results in enhanced signaling in tumor cells (17, 52, 53). Based on our studies, activation of Cdc42 may contribute to increased cancer invasion and metastasis by two complementary pathways, both of which are through the regulation of EGFR signaling. First, activation of Cdc42 inhibits c-Cbl-mediated ubiquitination and degradation of EGFR, resulting in sustained EGFR signaling (27). Second, this Cdc42-regulated increase in EGFR signaling likely contributes to the initial decrease in protein levels of E-cadherin at adherens junction by targeting E-cadherin for lysosomal degradation. The resulting destabilization of adherens junctions would then promote tumor progression toward more malignant states. The current studies establish a role for Cdc42 as a regulator of E-cadherin down-regulation and intracellular trafficking and further implicate Cdc42 as an important contributor to the invasive phenotype of human breast cancer cells.

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