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Phosphorylation of N-Cadherin-associated Cortactin by Fer Kinase Regulates N-Cadherin Mobility and Intercellular Adhesion Strength[V]

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Cortactin regulates the strength of nascent N-cadherin-mediated intercellular adhesions through a tyrosine phosphorylation-dependent mechanism. Currently, the functional significance of cortactin phosphorylation and the kinases responsible for the regulation of adhesion strength are not defined. We show that the nonreceptor tyrosine kinase Fer phosphorylates cadherin-associated cortactin and that this process is involved in mediating intercellular adhesion strength. In wild-type fibroblasts N-cadherin ligation-induced transient phosphorylation of Fer, indicating that junction formation activates Fer kinase. Tyrosine phosphorylation of cortactin after N-cadherin ligation was strongly reduced in fibroblasts expressing only catalytically inactive Fer (D743R), compared with wild-type cells. In wild-type cells, N-cadherin-coated bead pull-off assays induced fourfold greater endogenous N-cadherin association than in D743R cells. Fluorescence recovery after photobleaching showed that GFP-N-cadherin mobility at nascent contacts was 50% faster in wild-type than D743R cells. In shear wash-off assays, nascent intercellular adhesion strength was twofold higher in wild-type than D743R cells. Cortactin recruitment to adhesions was independent of Fer kinase activity, but was impacted by N-cadherin ligation-provoked Rac activation. We conclude that N-cadherin ligation induces Rac-dependent cortactin recruitment and Fer-dependent cortactin phosphorylation, which in turn promotes enhanced mobilization and interaction of surface expressed N-cadherin in contacting cells.

INTRODUCTION

Classical cadherins mediate intercellular adhesion in many tissues and impact multiple developmental and pathological processes. The association of cadherins with cortical actin filaments is required for the formation and maintenance of intercellular contacts (Adams *et al.*, 1996; Vasioukhin *et al.*, 2000). Actin filament remodeling at nascent, cadherin-mediated adhesions is mediated in part by the association of the Arp2/3 complex with the cytoplasmic domain of classical cadherins (Kovacs *et al.*, 2002b; Yap and Kovacs, 2003). Although the regulation of the strength and maturation of intercellular adhesions is poorly understood, local actin filament networks likely play crucial roles.

Several regulators of actin filaments associate with and strongly impact cadherin function and the formation of adherens junctions (Perez-Moreno *et al.*, 2003; Chan *et al.*, 2004). Notably, cortactin binds actin filaments and Arp2/3, and has recently been recognized as an important regulator of junction formation. Cortactin was originally discovered as a prominent substrate of the Src family of tyrosine kinases (Wu *et al.*, 1991) and later identified as a major organizer of cortical actin assembly, branching, and stability (Wu and Parsons, 1993; Weaver *et al.*, 2001), processes that are crucial for cell migration and invasion. Importantly, recent studies indicate that cortactin physically associates with nascent cadherin-mediated adhesions (El Sayegh *et al.*, 2004; Helwani *et al.*, 2004) and is required for their maturation and increased strength. In forming contacts, adhesion strength is a measure of the resistance of adhesions between adjoining cells to disruptive mechanical forces.

As cortactin is a well-known tyrosine kinase substrate, we considered that cortactin phosphorylation might impact the dynamics of intercellular contacts. Indeed, we have shown that the formation of strong, stable adhesions requires phosphorylation of tyrosines in the carboxy-terminus of cortactin, including tyrosine 421 (El Sayegh *et al.*, 2004). However, neither the responsible tyrosine kinase(s) nor the functional significance of cortactin tyrosine phosphorylation has been defined in the formation of cadherin-dependent contacts. We also showed that cortactin was recruited to sites of intercellular adhesion in a tyrosine phosphorylation-independent manner (El-Sayegh *et al.*, 2004); however, the underlying mechanism is unknown.

Here we explored the potential involvement of the nonreceptor tyrosine kinase Fer in phosphorylation of junctionally associated cortactin. We considered Fer is a possible

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candidate to catalyze this process for several reasons. First, although various tyrosine kinases can phosphorylate the three tyrosine residues in the carboxy-terminus of cortactin (Weed and Parsons, 2001), Fer physically associates with, and regulates N-cadherin junctions (Arregui *et al.*, 2000; Xu *et al.*, 2004). Moreover, cortactin is phosphorylated predominantly by Fer in response to different stimuli (Kim and Wong, 1998; Kapus *et al.*, 2000; Craig *et al.*, 2001), and embryonic fibroblasts derived from mice targeted with a kinase-inactivating mutation in fer exhibit large reductions of tyrosine phosphorylated cortactin (Craig *et al.*, 2001). Accordingly, we determined if N-cadherin ligation promoted Fer activation and if this process led to tyrosine phosphorylation of cortactin and increased adhesion strength. We also assessed if Fer kinase impacted the mobility and clustering of N-cadherin. Here we show that N-cadherin ligation activates the Fer kinase, a focal determinant for the ensuing cortactin phosphorylation and concomitant increased strength of nascent adhesions.

Further, we considered that N-cadherin ligation activates the small GTPase Rac, which may contribute to phosphorylation-independent recruitment of cortactin to N-cadherin contacts. This process has been implicated in the peripheral redistribution of cortactin induced by growth factors or mechanical stimuli (Weed *et al.*, 1998; Di Ciano *et al.*, 2002). Our results indicate that N-cadherin engagement activates Rac, which in turn promotes the recruitment of cortactin to N-cadherin junctions. Thus, N-cadherin ligation induces two separate signaling pathways that jointly lead to cortactin recruitment and phosphorylation; both of these processes are important for increasing the adhesion strength of nascent contacts.

MATERIALS AND METHODS

Cell Culture and Intercellular Adhesion

Rat-2 cells, mouse embryonic fibroblasts homozygous for a targeted catalytically inactivating Fer mutation (D743), or control wild-type fibroblasts were grown in DMEM supplemented with antibiotics and 5% (vol/vol) or 10% fetal bovine serum, respectively. To model intercellular adhesion processes we used the donor-acceptor model (Ko *et al.*, 2000) or plates or beads coated with a recombinant Ncad-Fc protein (El-Sayegh *et al.*, 2004). These model systems generate large numbers of synchronized, N-cadherin-mediated intercellular adhesions that facilitate biochemical and quantitative study with high temporal resolution. In the donor-acceptor model system, fibroblasts in suspension are added to the dorsal surfaces of substratum-bound, confluent monolayers of homotypic acceptor cells. The attachment of donor cells occurs via N-cadherin-mediated adherens junctions that are not confounded by integrin ligation to the substratum underlying the acceptor cells.

Immunoprecipitation and Immunoblotting

After treatments, samples were washed twice in phosphate-buffered saline (PBS) and extracted in ice-cold lysis buffer (100 mM NaCl, 30 mM HEPES, 2 mM AEBSF, 1 mM EDTA, 130 μM bestatin, 14 μM E-64, 1 μM leupeptin, 0.3 μM aprotinin, 1% Triton X-100, pH 7.5). After extraction, lysates were precleared for 1 h using 40 μl of 50% protein A/G-Sepharose bead suspension (Pierce Biotechnology, Rockford, IL). Lysates containing equal amounts of protein were incubated with primary antibodies recognizing N-cadherin (pan-cadherin antibody: Sigma, St. Louis, MO), cortactin (4F11; Upstate Biotechnology, Lake Placid, NY), Fer (anti-FerLA; Haigh *et al.*, 1996), pFer (rabbit polyclonal raised against the Fer activation loop phosphorylation site, QEDGGVpYSSSGLK; Bethyl Labs, Montgomery, TX), and protein-A/G Sepharose (Pierce Biotechnology) at 4°C for >1 h. Washed immunocomplexes were eluted from beads using 2% Laemmli sample buffer and boiled for 5 min. Samples were fractionated on 10% SDS-PAGE, transferred to nitrocellulose membranes, and blocked with 5% nonfat milk in 0.1% Tween 20 in Tris-buffered saline solution. Membranes were washed and immunoblotted for Fer (rabbit polyclonal anti-Fer; Haigh *et al.*, 1996), β-actin (E-5; Sigma), cortactin (Upstate), phospho-cortactin (Tyr 421, Tyr 486; AB 3853, AB3852; Chemicon, Temecula, CA), or phosphotyrosine (4G10; Upstate). Bound antibodies were detected with peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and chemiluminescence (Amersham, Oakville, ON, Canada).

RNA Interference and Preparation of GFP-tagged RNA Interference-resistant Constructs

A cortactin-specific small interfering (siRNA) oligonucleotide was transfected into cells as described elsewhere (El Sayegh *et al.*, 2004). Cells were assessed for cortactin protein content by Western blot and immunofluorescence. Experiments with cortactin-silenced cells were performed 24–48 h posttransfection. To generate GFP-cortactin proteins, the coding sequence of murine wild-type or F-cortactin (in which tyrosine residues 421, 466, and 482 had been replaced by phenylalanine) was inserted into pEGFP-C1 plasmid (BD Biosciences, Clontech, San Jose, CA) using the *Eco*RI and *Xho*I restriction sites. To obtain the siRNA-resistant versions of these same constructs, (WTr-GFP-cort or Fr-GFP-cort) two silent mutations were introduced at nucleotide 173 and 183 (C to T and T to C, respectively), using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according the manufacturer's instructions. These constructs have the same amino acid sequence as their siRNA-sensitive counterparts, but are not targeted with our siRNA against endogenous cortactin. In these experiments the cortactin siRNA oligonucleotide was transfected into cells as above, and 8 h later, samples were transfected with the GFP-cortactin siRNA-resistant constructs (FuGENE 6 Transfection Reagent, Roche, Laval, Québec, Canada). Experiments were performed 24 h after the latter transfection. Control experiments revealed that siRNA treated samples only expressed GFP-tagged siRNA-resistant versions of the cortactin constructs (unpublished data).

Preparation of N-Cadherin-Fc Beads and Dishes

The Ncad-Fc (chicken N-cadherin ectodomain fused to the Fc fragment of mouse IgG2b) protein was expressed in HEK-293 cells and collected as described (Lambert *et al.*, 2000). The following types of protein A-conjugated beads were used: magnetite, smooth-surface beads for pull-off assays (3 μm mean diameter, Spherotech, Libertyville, IL), polystyrene beads for cortactin spatial localization and wash-off assays (5 and 3 μm mean diameter, respectively; Spherotech). For coating of microbiological plastic plates, the N-cad Fc protein was isolated from conditioned media using Immunopure Plus immobilized protein G columns (Pierce Biotechnology) and exchanged into a sodium bicarbonate buffer (concentrations ~100 μg/ml). The protein was adsorbed onto plates by overnight incubation at 4°C and used immediately. Protein adsorption was quantified by dot blot and estimated at 1.25 $\mu g/cm^2$ based on densitometric comparisons with purified mouse IgG Fc fragment controls (Jackson ImmunoResearch Laboratories).

Spreading on Ncad-Fc Dishes

Cells harvested by gentle mechanical detachment, (cell scraper; Sarstedt, Newton, NC) to preserve endogenous surface expressed N-cadherin, were allowed to attach and spread onto N-cadherin-Fc-coated dishes for designated periods of time in complete growth media. After fixation, samples were stained with rhodamine phalloidin (Molecular Probes, Eugene, OR), and digitized images were analyzed to determine cell surface area (Compix Imaging systems, Cranberry Township, PA). Area values for experimental groups of Fer+/+ or D743R cells were normalized in each experiment to the sample group with the lowest mean area (assigned a value of 1).

Magnetic Bead Pull-off Assay and Bead-associated N-Cadherin Measurements

Cell surface proteins were enriched at sites of N-cadherin ligation with recombinant N-cadherin-Fc-coated beads. Bead-associated adhesion complexes were isolated and immunoblotted as described previously (Plopper and Ingber, 1993). Briefly, after designated incubation times, cells and attached N-cadherin-coated magnetic beads (Spherotech) were collected by scraping into ice-cold extraction buffer (CSK-EB: 0.5% Triton X-100, 50 nM NaCl, 300 mM sucrose, 3 mM Mg Cl_2, 2 mM AEBSF, 1 mM EDTA, 30 μM bestatin, 14 μM E-64, 1 μM leupeptin, 0.3 μM aprotinin, 10 mM PIPES, pH 6.8). Beads were pelleted using a side-pull magnetic isolation apparatus (Dynal, Lake Placid, NY) and supernatants were collected. Isolated beads were resuspended, sonicated, homogenized, and washed three times in extraction buffer before PAGE and Western blot analysis. For measurements of bead-associated endogenous N-cadherin, samples were incubated with beads in the presence or absence of cytochalasin D (1 μM). Bead-associated proteins were isolated, eluted and fractionated as above, and immunoblotted with an antibody to the cytoplasmic domain of N-cadherin to distinguish endogenous N-cadherin from the recombinant N-cadherin extracellular domain coating on the beads. Values for band densities were divided by the total number of beads bound per sample (as quantified by electronic particle counting) to estimate N-cadherin association.

Immunofluorescence

D743R and wild-type cells incubated with N-cad-Fc protein-coated beads were dual-stained for cortactin and Fer, or phospho-cortactin (pY421) and Fer. Samples were shear-washed at least four times in PBS, fixed with a 3.7% paraformaldehyde-5% sucrose solution, and permeabilized with 0.2% Triton-X. Samples were stained with rabbit polyclonal Fer antibody for 1 h at

37°C and counterstained with FITC-conjugated goat anti-rabbit Fab specific secondary antibody (Sigma; 1:100 at 37°C for 60 min). Samples were incubated either with Cy3 zenon-1-conjugated cortactin monoclonal antibodies or phosphocortactin antibodies (pY421) and counterstained with secondary antibodies for another hour at 37°C to permit visualization of total or phosphorylated cortactin. Samples were visualized with a Leica TCS SL confocal microscope (Deerfield, IL; excitation, 488 nm; emission, 520/10 nm for FITC). For Cy3 excitation was 543 nm and emission was 565/10 nm. Consecutive optical sections were obtained in the *z*-axis at a nominal thickness of 1 μm from the cell-substrate interface.

N-Cadherin-mediated Adhesion Strength

To estimate the relative strength and stability of intercellular adhesion Donor-acceptor cell preparations or N-cadherin-coated beads were subjected to shear forces by performing a logarithmic series of jet washes (Chou *et al.,* 1996). To assess the role of Fer in adhesion strength of newly formed contacts, D743R or D743R cells reconstituted with wild-type full length Fer-GFP (FuGENE 6; Roche) were compared with background matched wild-type cells. Ncad-Fc-coated polystyrene beads or donor cells were incubated with cells for 15 min. After jet washing, attached donor cells or bead associated samples were fixed as above. Attachment data were acquired from three, randomly chosen, low-power fluorescence microscopy fields. At least 30 cells were quantified per field and at least three fields were assessed per sample.

GTPase Activation

D743R and wild-type cells were seeded onto Ncad-Fc-coated 100-mm dishes, incubated at 37°C and harvested at 15, 30 and 120 min in lysis buffer with protease inhibitors (Upstate Biotechnology). Controls of each cell type were attached on poly-L-lysine (PL)-coated plates. Lysates were clarified by low-speed centrifugation. For rac and rho activation assays, supernatants were incubated with glutathione-agarose-bound GST fusion-proteins corresponding to the p21-binding domain of rac or the rhotekin-RBD, respectively. After 1 h of incubation at 4°C, samples were washed four times with buffer, boiled in Laemmli buffer, fractionated on 15% SDS-PAGE gels, transferred to nitrocellulose paper and probed with antibodies to rac (clone 23A8; Upstate Biotechnology) or to rho (clone 55; Upstate Biotechnology). Unfractionated supernatants were also separated and immunoblotted with the same rac and rho antibodies.

Live Cell Imaging and Fluorescence Recovery after Photobleaching

For tracking intercellular adhesion formation or fluorescence recovery after photobleaching (FRAP) analysis, Rat-2 or the Fer +/+ and D743R cells were seeded onto 35-mm fibronectin-coated glass bottom microwell dishes (MatTek Corporation, Ashland, MA) overnight before transfection with a full-length N-cadherin EGFP construct obtained from C. Gauthier-Rouviere (Mary *et al.,* 2002). On achieving desired confluence, samples were washed and replenished with fresh growth media (α-MEM without HCO_3^- containing 25 mM HEPES buffer and serum). Samples were placed on a heated stage and imaged using a confocal microscope set with a scan speed set at 400 or 800 Hz, respectively and settings as described for FITC fluorescence above. For video-microscopy, regions of imminent intercellular contact were imaged every 60 s for up to 6 h.

FRAP experiments were conducted on intercellular contacts identified as nascent or mature as characterized by videomicroscopic analysis. Circular regions of interest 3.8 μm in size were photobleached at 32 times optical zoom (40× objective) for 1.7 s with a high-intensity laser (150–250 mW), and recovery was observed postbleaching continuously for 600 s. Analysis was conducted by converting fluorescence output data to relative fluorescence by adjusting for total sample bleaching during recovery in control regions peripheral to the experimentally bleached region. Control regions of similar starting fluorescence intensity to that of the experimental region were either taken from noncontacting zones or from another contact area in the field of view. The % mobile fraction and the diffusion coefficient were computed as described (Arora *et al.,* 2004).

Statistical Analysis

For continuous variables, means and SEs of the mean were computed. Comparisons between two groups were made with the unpaired Student's *t* test and multiple comparisons by ANOVA. Statistical significance was set at $p < 0.05$.

RESULTS

Fer Is Required for N-Cadherin-dependent Tyrosine Phosphorylation of Cortactin

We first evaluated the role of Fer in the phosphorylation of cortactin after N-cadherin ligation by comparing this event in fibroblasts from wild-type embryos, or those with a targeted homozygous kinase-inactivating and protein-destabilizing missense mutation (D743R) in the *fer* locus (Craig *et al.,* 2001). Wild type (+/+) or D743R (−/−) cells were attached to nontissue culture substrata coated with recombinant Ncad-Fc. After defined incubation times, cell lysates were prepared, cortactin was immunoprecipitated and its phosphorylation status was evaluated by immunoblotting with anti-phosphotyrosine antibodies (4G10). We found time-dependent tyrosine phosphorylation of a protein corresponding to the molecular mass of cortactin; the intensity of this signal peaked at 30 min and decayed thereafter (Figure 1A, 4G10, 15+/+, 30+/+, and 60+/+ samples). To verify that the phosphorylated protein was indeed cortactin, the blots were stripped and reprobed with antibodies generated against two major cortactin tyrosine phosphorylation sites at Y421 or Y486. Both antibodies detected temporally similar cortactin-phosphorylation patterns as described above (Figure 1A, pY421 and pY486, 15+/+, 30+/+, and 60+/+). We ensured that this was a ligand-specific cortactin-phosphorylation event by performing parallel analysis of cells plated on PL-coated dishes for 30 min. There was no detectable tyrosine phosphorylation of cortactin under these conditions (Figure 1A, PL +/+, PL −/− samples). In contrast to the observations in Fer wild-type cells, D743R cells exhibited very low levels of cortactin tyrosine phosphorylation in response to N-cadherin ligation (Figure 1A, 15−/−, 30−/−, and 60−/− samples). Notably, a very small proportion of cortactin was phosphorylated in the D743R cells after the formation of N-cadherin adhesions, suggesting that other kinases may also play a role in cortactin phosphorylation, although their relative contributions are apparently minor.

We next evaluated the phosphorylation state of the pool of cortactin that was associated with N-cadherin adhesions. Ncad-Fc-coated magnetic beads were attached to a monolayer of Fer wild-type or D743R cells, and bead-associated proteins were isolated and assessed by immunoblotting. Consistent with the results described above, there was a temporally similar pattern of cortactin tyrosine phosphorylation in which maximal levels of tyrosine phosphorylation were detected at 30 min followed by a decrease thereafter (Figure 1B). In comparison with wild-type cells, proteins eluted from beads incubated with D743R cells showed very low levels of cortactin tyrosine phosphorylation.

To verify the biochemical results obtained above, we conducted immunofluorescence analysis using confocal microscopy with either the donor-acceptor model or N-cadherin-coated beads. In support of biochemical analysis, immunofluorescence analysis of phosphorylated cortactin (pY 421) showed prominent staining around Ncad-Fc beads in wild-type cells, whereas D743R cells exhibited substantially less staining at the bead periphery (Figure 1C, i–vi). Similarly using the donor-acceptor model (Figure 1C, vii–xiv), wild-type donor cells exhibited pY cortactin staining that was peripherally localized and dramatically greater in intensity (viii) than the diffuse staining seen in D743R cells (xii). In contrast, the underlying acceptor monolayer of cells demonstrates approximately equivalent staining intensity and distribution in both cell types (Figure 1C, ix and xiii). Importantly, transient transfection of a full-length Fer-GFP into the D743R cells restored the phosphorylated cortactin staining in regions of the Ncad-Fc bead periphery (Figure 1D) and caused a general increase in the PY-cortactin labeling. Collectively, these results implicate the Fer kinase as a major determinant of adhesion-associated tyrosine phosphorylation of cortactin.

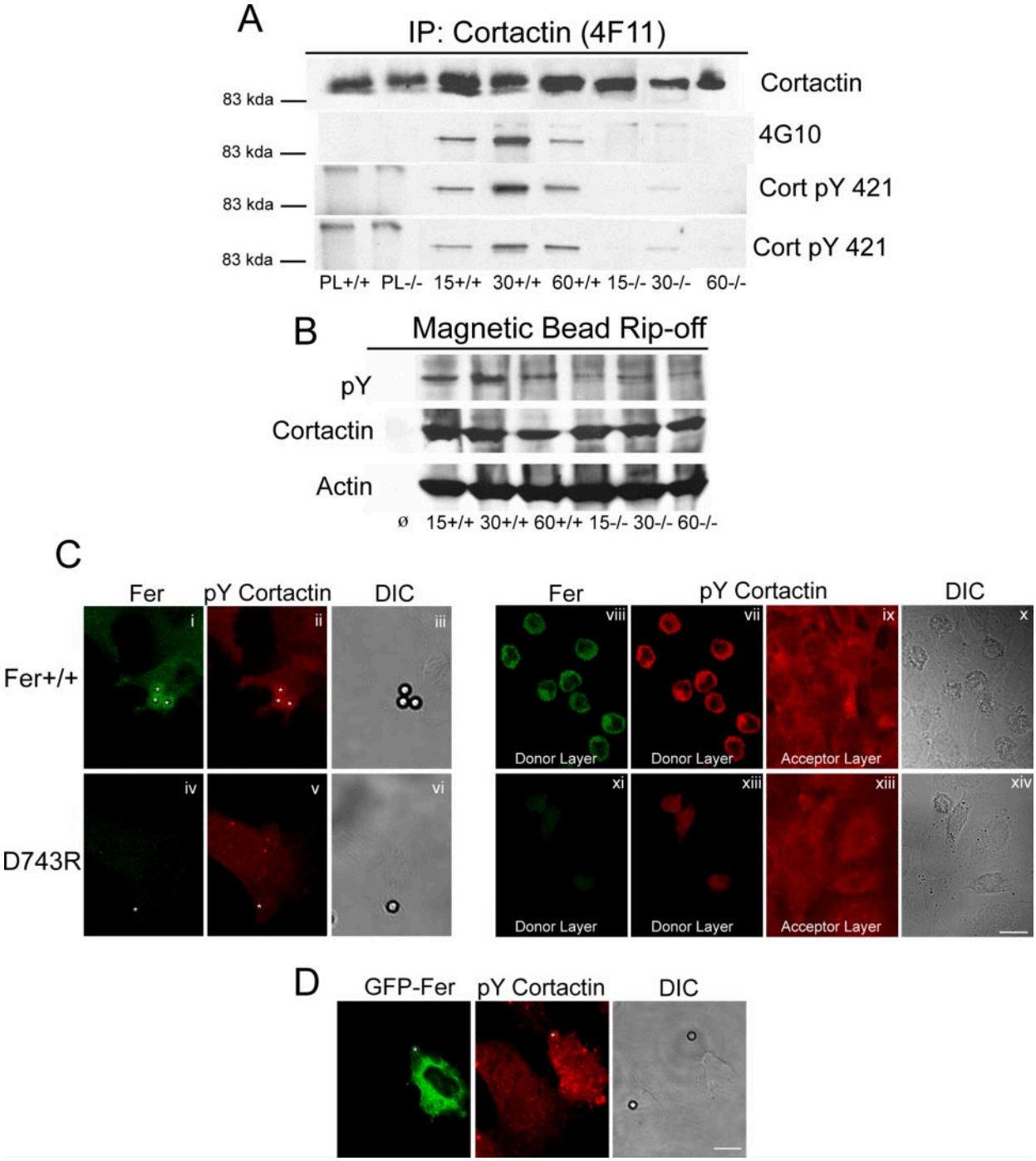

Figure 1. Reduced cortactin tyrosine phosphorylation upon N-cadherin ligation in D743R fibroblasts. (A) Wild-type (+/+) or D743R (−/−) cells were allowed to attach to N-cad-Fc-coated bacteria-grade Petri-dishes or poly-L-lysine (PL)-treated tissue-culture dishes for 15, 30, or 60 min. Cell lysates were prepared and cortactin was immunoprecipitated (IP) and subjected to sequential immunoblotting analysis using antibodies to cortactin (SCBT H-191), phosphotyrosine (4G10), or phospho-cortactin recognizing pY421 (CortPY421) or pY486 (CortPY486). (B) Ncad-Fc-coated magnetic beads were applied to wild-type (+/+) or D743R (−/−) cells for 15, 30, or 60 min. N-cad-associated proteins were then isolated from cell lysates as described in *Materials and Methods* and assessed by immunoblotting with antibodies to phosphotyrosine (pY), cortactin or β-actin. (C) (i–vi) Ncad-Fc-coated beads or (vii–xiv) homotypic donor cells were applied to underlying wild-type (+/+) or D743R (−/−) cells for 15 min. Cells were fixed and subjected to immunofluorescence staining for Fer or pY421 cortactin, and the position of the beads or donor cells was determined by differential interference contrast (DIC) microscopy and or *z*-axis-oriented optical sectioning. Optical sections of donor cells (vii, viii, xi, and xii) or confluent underlying acceptor monolayers (ix and xiii). Results shown in i–vi are representative of ~90% of bead-bound cells (42 and 39 of 45 examined beads bound wild-type and D743R cells, respectively), and (vii–xiv) 100% of observed bound donor cells {90 cells evaluated for each cell type). (D) Ncad-Fc-coated beads were applied for 15 min to D743R cells transfected with Fer-GFP. Fer-GFP was detected by direct fluorescence, pY421 was revealed by immunofluorescence and the position of the beads was revealed by DIC microscopy. These results are characteristic of ~80% of the Fer-GFP-transfected cells (24 out of 30 examined positively transfected cells). Bars, 10 μm

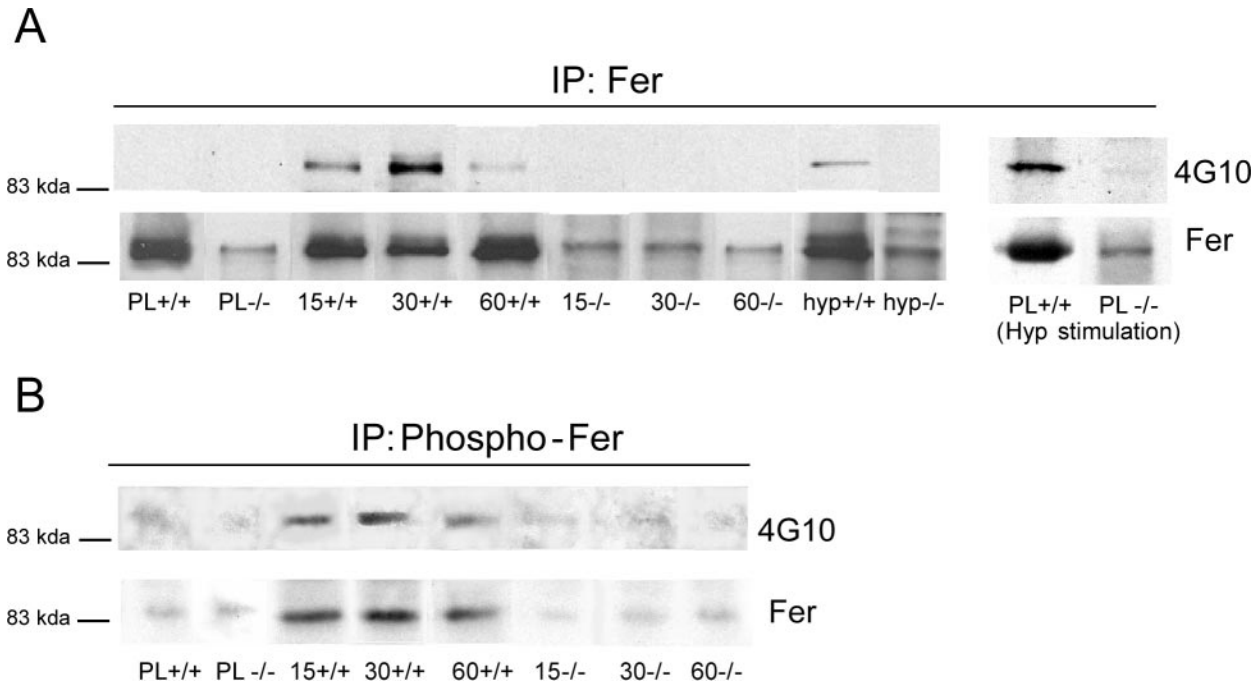

Figure 2. Fer is tyrosine phosphorylated upon N-cadherin ligation. (A and B) Wild-type (+/+) or D743R (−/−) cells were attached to N-cad-Fc-coated Petri-dishes or poly-L-lysine (PL)-treated tissue-culture dishes for 15, 30, or 60 min and were subsequently treated with hyperosmotic media as indicated (hyp). Cell lysates were prepared and immunoprecipitated with antibodies to Fer or to phospho-Fer. Immunoprecipitates were immunoblotted with antibodies to phosphotyrosine (4G10) or Fer, as indicated.

N-Cadherin Ligation Induces Tyrosine Phosphorylation of Fer at Nascent Contacts

Fer associates with N-cadherin (Arregui *et al.*, 2000) through an interaction with the N-cadherin-binding protein p120-catenin (Xu *et al.*, 2004), but it is not known if N-cadherin ligation is an activating signal for Fer. We examined tyrosine phosphorylation of Fer at nascent intercellular contacts as a surrogate for Fer kinase activation (Ben-Dor *et al.*, 1999; Greer, 2002). Wild-type and D743R cells were attached to Ncad-Fc or PL-coated substrata, and Fer immunoprecipitates were immunoblotted for phosphotyrosine. N-cadherin ligation promoted tyrosine phosphorylation of Fer within 15 min in wild-type cells, which peaked at 30 min, followed by a large reduction at 60 min (Figure 2A). There was no detectable tyrosine phosphorylation of Fer in wild-type cells attached to PL, indicating an N-cadherin-specific response (Figure 2A, PL+/+, PL−/− samples). To validate this control and to assess whether Fer retained the potential to be activated under these conditions, cells were attached to PL-coated substrata and subjected to hyperosmotic stress, a procedure that activates Fer-independent of cell attachment (Kapus *et al.*, 2000). As expected, hyperosmotic stress-induced significant tyrosine phosphorylation of Fer in wild-type cells attached to PL, indicating that the signaling pathways converging on Fer remained intact in cells anchored to PL substrates (Figure 2A, last two lanes). Although a small amount of Fer protein was immunoprecipitated from D743R cells, it showed no detectable phosphorylation.

We substantiated these findings by immunoprecipitating Fer with an antibody raised against the activation loop phosphorylation site, which is specific for activated Fer kinase (anti-phospho-Fer). Lysates were prepared from wild-type and D743R cells that had attached to either Ncad-Fc or PL substrates. Anti-phospho-Fer immunoprecipitates were first immunoblotted for phosphotyrosine and then stripped and reprobed with polyclonal Fer antibody (Figure 2B). Phosphorylated Fer was detected exclusively in immunoprecipitates from wild-type cells, and the kinetics of phosphorylation were similar to that seen in data obtained with the reagents described in Figure 2A. Phosphorylated Fer was not detected in lysates of wild-type cells attached to PL, confirming a specific role for N-cadherin in inducing this response. Hypertonic treatment stimulated Fer phosphorylation in wild-type cells attached to PL, indicating that Fer could be activated in these cells (unpublished data). As expected, no phosphorylated Fer was detected in D743R cells (Figure 2B). Thus N-cadherin ligation induces phosphorylation of Fer kinase, a transient response that temporally coincides with tyrosine phosphorylation of adhesion-associated cortactin.

Fer Kinase Activity Is Required for the Formation of Strong Intercellular Adhesions

A well quantifiable measure of adhesion strength is the capacity of the adhesive structures to resist the detachment of cadherin-coated particles under flow and shear stress (Yap *et al.*, 1997; Chan *et al.*, 2004; El Sayegh *et al.*, 2004). To evaluate the role of Fer kinase activity in the strength/stability of newly formed N-cadherin-mediated intercellular adhesions, shear wash-off assays were conducted with donor-acceptor cultures of wild-type or D743R cells in which donor cells were attached to a completely confluent, underlying homotypic acceptor monolayer for a period of 15 min before initiating the wash series. D743R cells exhibited a twofold reduction in the number of attached donor cells at all points during the wash series ($p < 0.01$; Figure 3A). In-depth analysis of this data set showed that the initial stability of donor-acceptor cell interactions is reduced in the D743R cells: there is a ~60% reduction in the number of D743R cells between the first two wash-off points compared

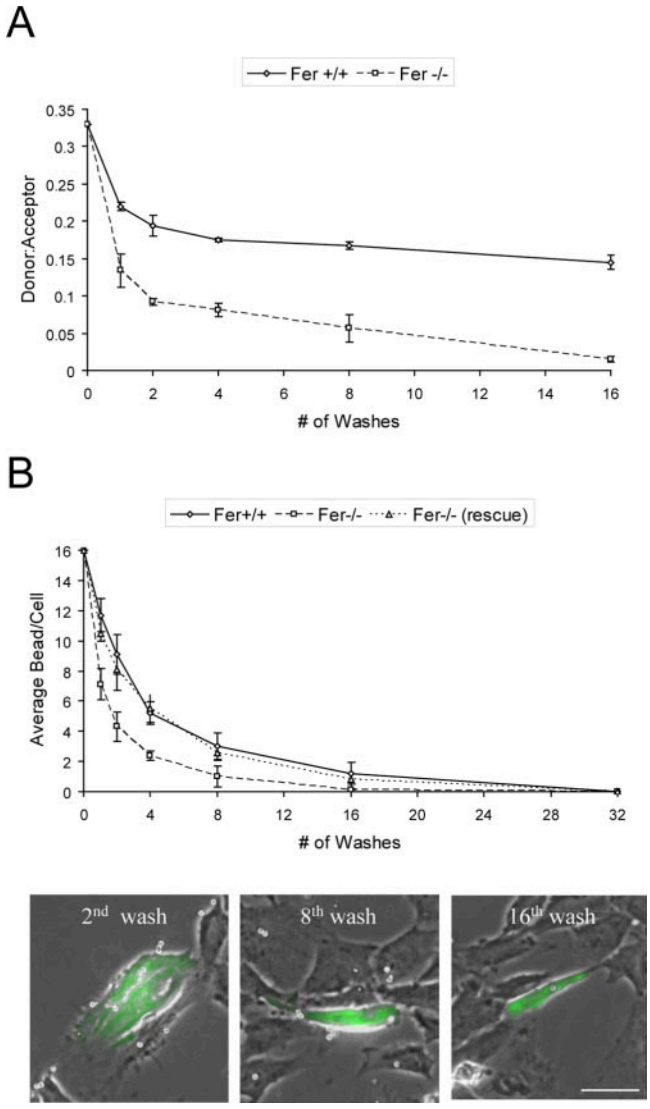

Figure 3. Fer kinase activity is required for the development of adhesion strength in newly formed N-cadherin-mediated contacts. (A) Labeled donor wild-type (+/+) or D743R (−/−) cells were seeded onto confluent acceptor monolayers of the same genotype for 15 min, and adhesion strength was assessed by quantifying attached cells after washing as indicated. (B) Ncad-Fc-coated beads were applied onto monolayers of wild-type (+/+), D743R (−/−), or D743R cells transfected with Fer-GFP (rescue) for 15 min. Beads associated with cells were then quantified after washes as indicated by DIC microscopy or combined fluorescence and DIC microscopy in the case of the rescue cells. Bar, 20 μm.

with the 33% reduction in Fer +/+ cells (Figure 3A, D743R: 0.33–0.134, Fer +/+: 0.33–0.22). Moreover, the strength of the initially formed contacts (i.e., those present after the second wash) is far less in the D743R cells because 88% of these contacts are lost over the span of the wash-off series compared with 34% in the Fer+/+ cells (Figure 3A, D743R: 0.134–0.016, Fer +/+ 0.22–0.145). Conversely, there is a 5.5-fold greater proportion of adherent Fer +/+ donor cells than D743R cells at the end of the wash series (Figure 3A, 12 and 66% of D743R and Fer+/+ cells remained attached, respectively, at wash 16).

A role for Fer kinase function in regulating N-cadherin-mediated adhesion strength was more definitively established using D743R cells reconstituted with a transfected Fer-GFP construct. In these experiments cells were incubated with Ncad-Fc-coated beads and the number of beads associated with the GFP-positive transfected cells were quantified by microscopy as a function of the number of washing steps. The expression of Fer-GFP corrected the intercellular adhesion defect observed in the previous experiment, because the reconstituted cells demonstrated adhesion strength that was not significantly different from the level observed in wild-type cells (Figure 3B). These results indicate that Fer kinase activity is important for regulating nascent N-cadherin-mediated intercellular adhesion strength.

Fer Promotes the Binding of Endogenous N-Cadherin to N-Cadherin-coated Beads

Conceivably, Fer kinase might contribute to adhesion strength by increasing the association between N-cadherin and the actin cytoskeleton and/or by promoting more efficient cadherin/cadherin interactions between neighboring (cell) surfaces. Surface organizational changes that enhance cadherin engagement, most notably increased lateral clustering, has been described as an efficient means to strengthen cadherin-dependent adhesions (Yap *et al.*, 1997). To examine these possibilities, initially we determined if Fer impacts the total amount of N-cadherin-associated actin. We conducted Ncad-Fc-coated bead pull-off assays on wild-type and D743R cells and found that equivalent amounts of β-actin were associated with the beads from both cell types over the time course (Figure 4A). Because previous studies have demonstrated that cadherin clustering plays a fundamental role in mediating intercellular adhesion strength (Yap *et al.*, 1997) and the cadherin expression profile of wild-type and D743R cells is known to be equivalent (Xu *et al.*, 2004), we next asked if there was a defect in cell surface organization of cadherin in D743R cells in response to N-cadherin ligation. N-cad-Fc-coated magnetite beads were attached to monolayers of D743R and wild-type cells, and bead-associated endogenous N-cadherin was assessed by immunoblotting over a time course. Blot densities were normalized in each sample to the total number of bound beads (see *Materials and Methods*), which allowed direct comparisons of the total amount of cell surface-expressed N-cadherin/bead. There was a nearly fourfold increase in bead-associated N-cadherin in wild-type cells between 15 and 30 min with no change at 60 min ($p < 0.001$; Figure 4B). As the number of bound beads per cell increased marginally from 15 to 30 min (unpublished data), these data indicate a substantial degree of ligand-induced organizational changes suggestive of lateral clustering during this interval. In contrast, D743R cells showed no significant change in bead associated N-cadherin over the time course. To determine if the increase of bead-associated N-cadherin observed in wild-type cells was dependent on intact actin filaments, parallel analyses were also conducted on cytochalasin D-treated cells. It is noteworthy that under these conditions the previously noted increase in bead-associated N-cadherin of Fer+/+ cells was not observed (Figure 4B). In addition, the total amount of protein eluted and the number of bound beads was somewhat reduced.

To determine the potential role of cortactin in ligand-induced N-cadherin organizational changes, cortactin expression was knocked down in Fer wild-type and D743R cells using siRNA-mediated gene silencing (see *Materials and Methods*). Approximately 90% knockdown was achieved in both the Fer +/+ and D743R cells as detected by Western blotting (Figure 4C). Wild-type cortactin knockdown sam-

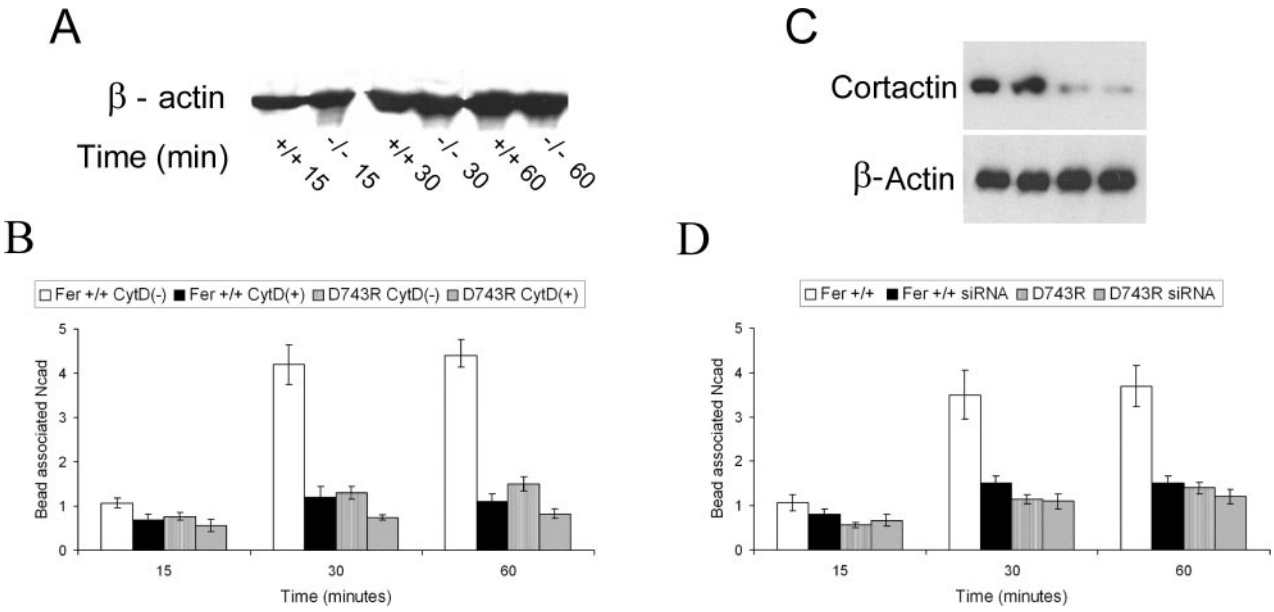

Figure 4. Fer kinase influences actin-dependent cell surface organization of N-cadherin upon ligation. (A) Ncad-Fc-coated magnetic beads were applied to wild-type (+/+) or D743R (−/−) cells for 15, 30, or 60 min, and bead-associated actin was assessed as described in *Materials and Methods*. (B) The magnetic bead rip-off assay described above was repeated with cells treated with cytochalasin D or not, as indicated. Bead-associated N-cadherin was quantified and expressed relative to that seen in wild-type cells at 15 min in the absence of cytochalasin D. (C) Wild-type (+) or D743R (−) cells were treated with vehicle (V) or a specific RNAi against cortactin (S) as described in *Materials and Methods*. The cortactin content of whole cell lysates was assessed by Western blotting. (D) Magnetic bead rip-off assay in cortactin RNAi or vehicle control-treated samples. Bead-associated N-cadherin was quantified and expressed relative to that seen in wild-type cells at 15 min in vehicle controls.

ples demonstrated dramatically reduced levels of bead-associated N-cadherin when compared with vehicle controls (Figure 4D). Cortactin knockdown eliminated the differences seen in vehicle control samples of D743R and wild-type cells, implying that the interaction between Fer kinase and cortactin was responsible for promoting N-cadherin surface organizational changes (Figure 4D). Cortactin knockdown in D743R cells did not effect the levels of bead-associated N-cadherin (Figure 4D). Collectively these data indicate that the association of cadherin adhesion complexes with the actin cytoskeleton is not dependent on Fer kinase activity but that ligand induced N-cadherin organizational changes requires intact actin filaments, Fer kinase activity, and the substrate for this kinase, cortactin.

Fer Kinase Activity Influences N-Cadherin Mobility at Nascent Contacts

The evidence described above for a defect in cell surface N-cadherin organization suggestive of compromised lateral clustering in D743R cells indicates a role for Fer in the mobility of N-cadherin in forming cell contacts. To evaluate this possibility, we conducted a FRAP analysis of nascent contacts of cells transfected with a full-length N-cadherin-GFP construct. However, a significant difficulty with this approach is that very little is known about the kinetics of N-cadherin-mediated contact formation and the accompanying morphological characteristics. Because contacts engaged in different phases of junction formation may exhibit different average mobilities, initially we sought to characterize the process and establish morphological hallmarks for the various phases of junction dynamics. To achieve this, we acquired numerous long time-span live videos of N-cadherin-GFP-transfected Rat-2 fibroblasts and analyzed the typical morphology of these contacts throughout the progression of contact maturation as has been done previously for E-cadherin (Adams *et al.*, 1998). We found that compared with E-cadherin-mediated contacts, N-cadherin contacts are more dynamic structures and exhibit greater instability. Nascent contacts are characterized by an orientation of N-cadherin molecules radial to the plane of the contact (Figure 5, i and iv, white arrow). These contacts are unstable and may exhibit continued remodeling with cycles of contact formation and dissolution, or contact extension and stabilization (Figure 5, red vs. white arrows, see Supplementary Video: Figure 5.avi). Contact maturation is characterized by a belt-like orientation of N-cadherin molecules parallel to the plane of contact in addition to extension of the contact zone, which is facilitated by highly mobile peripheral clusters of N-cadherin (Figure 5iii, yellow diamond arrow).

Qualitatively, N-cadherin-GFP-transfected wild-type and D743R cells displayed very similar patterns of junctional formation and maturation as described above (see micrographs, Figure 6A, i–iii). However, FRAP analysis of wild-type and D743R cells demonstrating the typical radial morphology of nascent contacts suggested that both the initial rate of recovery and the extent of recovery were greater in wild-type cells (Figure 6Ai, zones 1 and 3). In contrast, mature junctions displayed similar rates and extent of recovery of fluorescence between the two cell types (Figure 6Bii). Remarkably, the percentage of total recovery in mature junctions was consistently only slightly lower than that seen in nascent contacts (Figure 6Aii, zone 3–85%), in contrast to the situation observed in E-cadherin-mediated junctions (Adams *et al.*, 1998). FRAP of clusters of N-cadherin on the periphery of mature zones of contact demonstrated similar kinetics in both cell types with a recovery rate intermediate to both nascent and mature junctions. These clusters exhibited the most complete and rapid recovery after bleach-

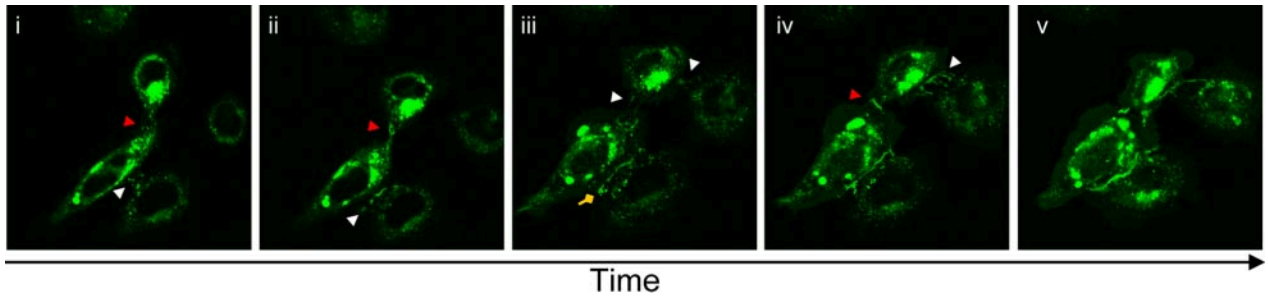

Figure 5. N-cadherin-mediated intercellular contacts formation: Full-length N-cad-GFP-transfected Rat-2 cells in close proximity over an 8-h time course reveal the dynamic nature of contact formation and instability. Initial contact formation indicated by white arrows in i) and iii). Red arrow indicates a continuously remodeling unstable contact while white arrows (i, ii, and iv) indicate position of progressive contact formation. (i and iv) Radial pattern of N-cadherin at nascent contacts; ii) contact zone extension and stabilization (white arrow) and concurrent remodeling and loss of contact formation (red arrow); (iii) two zones of nascent contact formation (white arrows) and highly mobile peripheral cluster, which extends through contact zone (yellow diamond arrow); (iv) radial pattern representative of nascent contact formation (white arrow); (v) mobile cluster extends through contact zone. Red arrow indicates continued remodeling and junction instability.

ing (Figure 6Aiii, recovery is complete in early zone 2), consistent with their high mobility (see Supplementary Video: Figure 5.avi).

Because the impact of Fer kinase activity on contact dynamics was highly dependent on their maturation state, we examined N-cadherin mobility under conditions when contact formation can be more predictably synchronized. This process was quantified in a well-defined early time period using Ncad-Fc-coated substrata. Wild-type and D743R cells were allowed to attach for 30 min to Ncad-Fc-coated substrata and FRAP analysis was conducted on regions of the cell periphery corresponding to areas of nascent contact zone extension. Wild-type cells demonstrated a 2.4-fold higher diffusion coefficient and a 47% greater maximal recovery in regions of nascent contact formation compared with kinase-dead cells ($p < 0.001$, Figure 6B), supporting the result obtained with native intercellular contacts. In conclusion, these data correspond well with the dynamic nature of contact formation observed above and indicate that N-cadherin mobility at nascent junctions is regulated by Fer activity.

Adhesion Zone Extension Is Dependent on Fer Kinase Activity

We asked if in addition to regulating N-cadherin mobility at nascent contacts, Fer kinase activity might be required for more rapid contact formation and contact zone extension, a question that can be efficiently addressed using the Ncad-Fc-coated substratum model. Wild-type and D743R cells were allowed to attach and spread on Ncad-Fc or fibronectin (control)-coated substrata for 30 and 180 min and analyzed for total spreading area. Indeed we noted a greater than twofold increase in the mean spreading area of wild-type compared with D743R cells at both the 30 and 180 min time points on Ncad-Fc-coated surfaces (Figure 7A). Moreover, this effect was substratum-specific, because this trend was reversed on fibronectin, where this substrate the kinase-dead cells exhibited faster spreading than the wild-type cells (Figure 7A).

To definitively evaluate if the observed differences in N-cadherin contact zone extension were related to Fer-mediated cortactin tyrosine phosphorylation, wild-type and D743R cells were treated with RNA interference (RNAi) to knock-down endogenous cortactin and rescued with GFP tagged RNAi-resistant wild-type (WT-cort) cortactin, or a full-length construct in which tyrosine residues 421, 466, 482 were mutated to phenylalanines (F-cort). Cells were then allowed to attach and spread on Ncad-Fc-coated substrata as described above. At both 30 and 180 min that there was a nearly twofold difference in mean spreading area between the Fer +/+ cells transfected with Wt-cort and F-cort constructs (Figure 7B). No differences were noted in D743R cells transfected with either construct (Figure 7B) as compared with the nontransfected cells.

Collectively these data suggest that Fer-mediated cortactin tyrosine phosphorylation leads to a faster rate of N-cadherin contact zone extension, and impacts on the adhesion strength of the nascent contacts.

Cortactin Recruitment Is Independent of Fer

We determined if cortactin recruitment to N-cadherin-mediated intercellular adhesions was dependent on Fer. Ncad-Fc-coated beads were attached to monolayers of wild-type and D743R cells. Bead-associated proteins were extracted as described previously, normalized for protein concentration and immunoblotted for cortactin. Equivalent amounts of cortactin were recruited to N-cadherin adhesions in wild-type and D743R cells, and total expression levels of cortactin were equivalent in the two cell types (Figure 8A). Confocal microscopy showed that Ncad-Fc-coated beads induced accumulation of both Fer and cortactin around the bead periphery, and cortactin accumulation was similar in D743R and wild-type cells (Figure 8B). These findings indicate that cortactin recruitment is independent of Fer kinase activity and support our previous study showing that cortactin recruitment to N-cadherin junctions is independent of tyrosine phosphorylation (El-Sayegh *et al.,* 2004). Further, because the levels of kinase-inactive Fer expressed in D743R cells are greatly reduced because of instability of the mutant protein (Craig *et al.,* 2001), this result also suggests that a kinase-independent function of Fer is not involved in cortactin recruitment.

N-Cadherin-mediated Rac Activation Recruits Cortactin

Rho family GTPase activation mediates the translocation of cortactin from the cytoplasm to membrane ruffles (Weed *et al.,* 1998). We next asked if loss of Fer impacts Rho family GTPase activation and cortactin recruitment to N-cadherin adhesions. To test whether N-cad ligation induces small GTPase activation in this cellular context, we used Ncad-Fc-coated nontissue culture substrata to eliminate the possible confounding effects of integrin ligation. In wild-type cells, Rac activation was transiently enhanced by N-cadherin ligation, but this response dissipated within 30 min. In con-

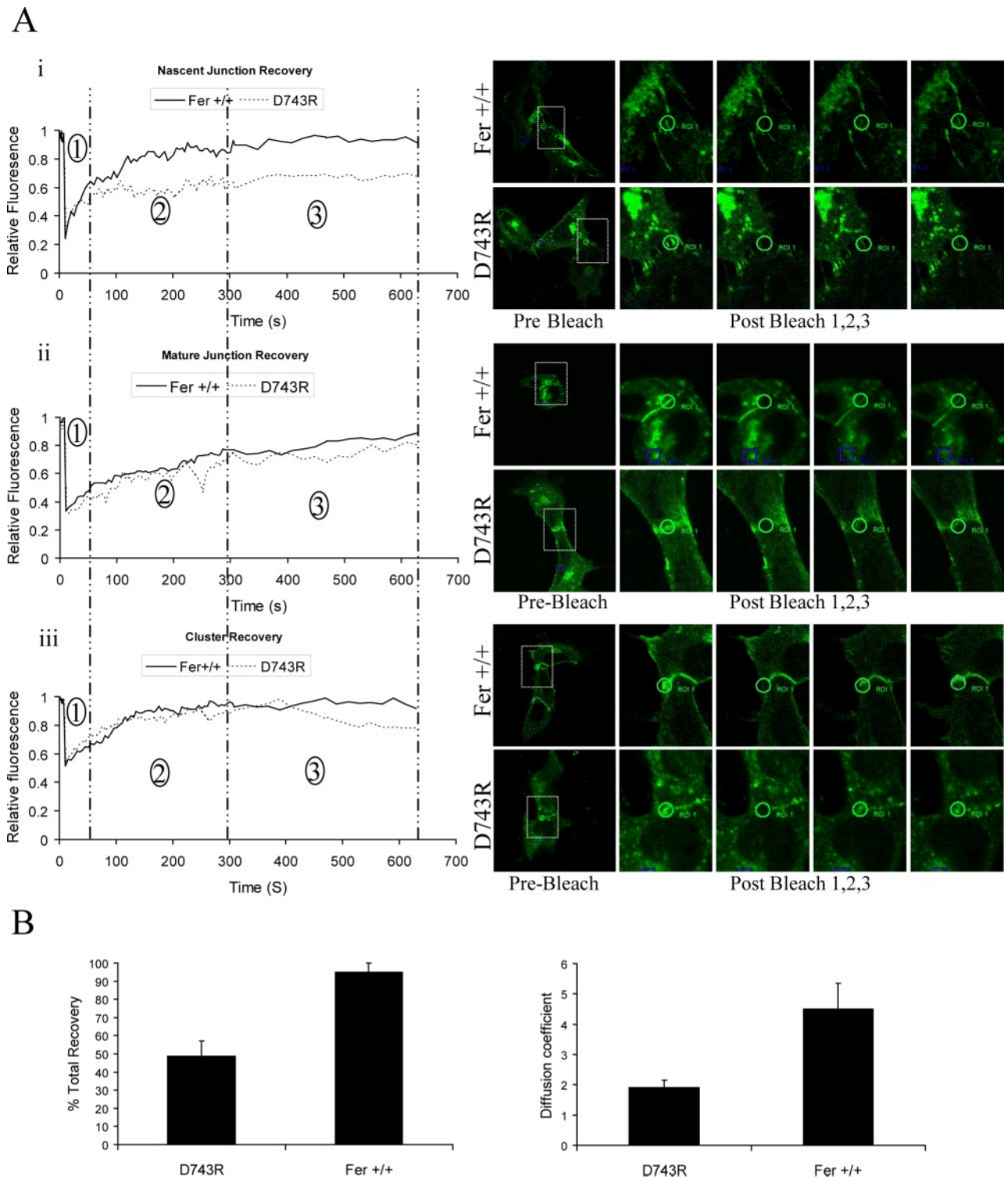

Figure 6. Fer kinase activity influences N-cadherin mobility. (A) FRAP of N-cad-GFP in transfected wild-type and D743R cells for nascent (i) and mature (ii) junctions in 3.8-μm circular regions (shown in green on micrographs). Typical relative fluorescence plots (left) and corresponding micrographs (right) are presented for each experiment. Control (unbleached) regions in micrographs are in blue squares. (B) Quantification of FRAP analysis of wild-type and D743R cells bound to N-cad-Fc-coated substrata in zones of contact extension reveals significantly higher rate ($p < 0.001$) (right), and total recovery of fluorescence (left) in wild-type cells.

trast, Rho activation was not detectable at early times after ligation (representing nascent junction formation) but was evident at later time points (representing more mature junctions; Figure 8C). In D743R cells we found temporal and sequential activation of Rac and Rho similar to that seen in wild-type cells, indicating that Rac and Rho GTPase activa-

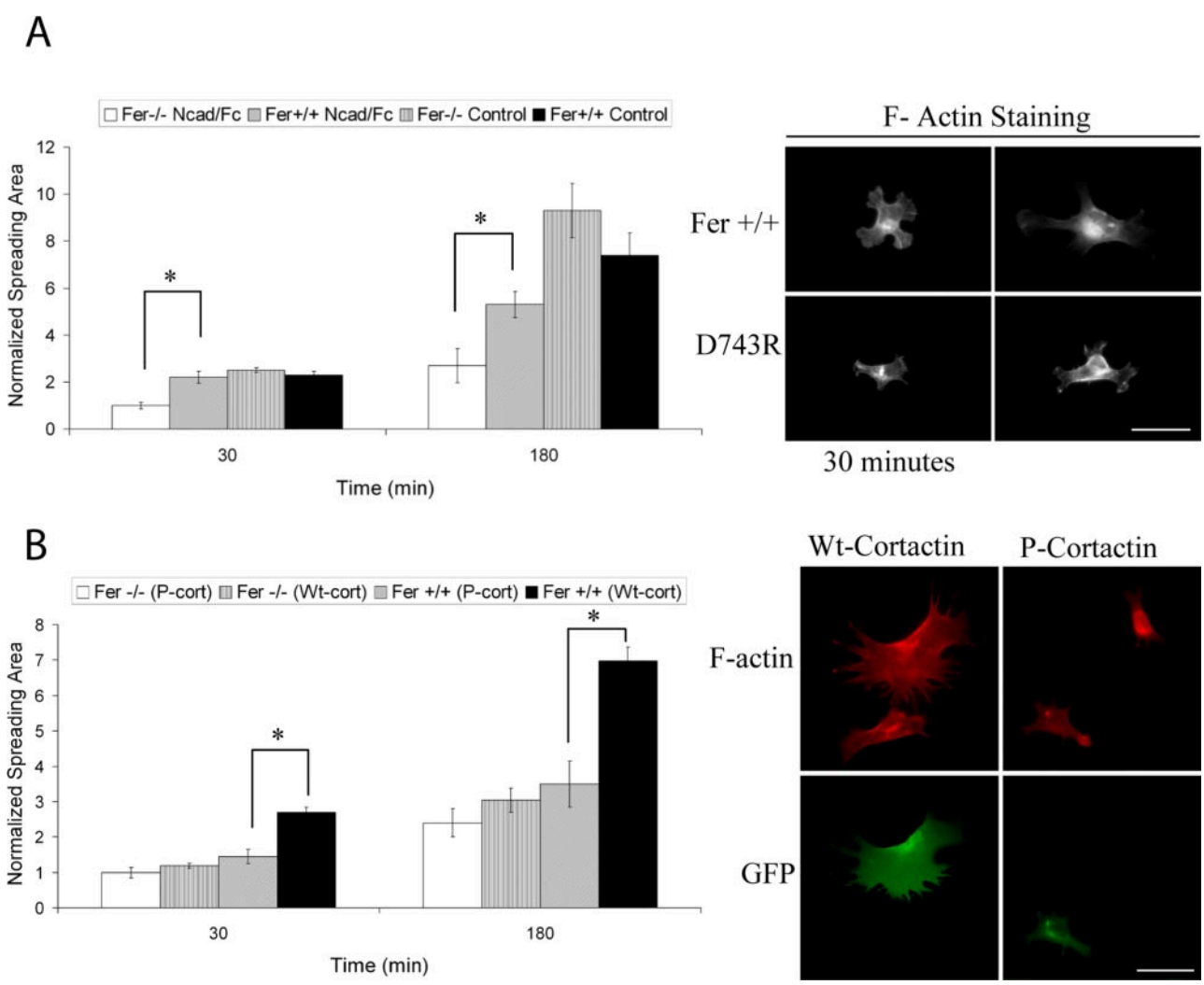

Figure 7. Fer kinase activity is required for efficient contact zone extension on an N-cadherin-based substrate. (A) Spreading of wild-type and D743R cells on N-cad-Fc or fibronectin-coated surfaces was quantified at 30 and 180 min as described in *Materials and Methods* (left). Images of F-actin-stained wild-type and D743 cells spreading on Ncad-Fc surfaces at 30 and 180 min are shown on right. (B) Spreading of wild-type and D743R cells depleted of endogenous cortactin by RNAi and concomitantly transfected with RNAi-resistant GFP-tagged cortactin constructs on N-cad-Fc-coated substrata as quantified at 30 and 180 min. Wild type (WT-cort) cortactin or a construct in which tyrosine residues 421, 466, and 482 were mutated to phenylalanines (F-cort) were used. Images of Fer+/+ cells at 180 min. *$p < 0.05$ between groups. Bars, 20 μm.

tion upon N-cadherin ligation is independent of Fer kinase function. No Cdc42 activation was detected in either wild-type or D743R cells in response to N-cadherin ligation (unpublished data).

We also investigated the effect of Rac activation on cortactin recruitment to nascent N-cadherin-mediated adhesions using biochemical methods. Cells were transfected with GFP-tagged wild-type and dominant negative Rac constructs. Transfected cells were sorted by flow cytometry and attached to Ncad-Fc-coated substrata for 20 min. Lysates of normalized protein concentrations were prepared from the adherent cells. N-cadherin was immunoprecipitated, and cortactin association was assessed by immunoblotting analysis. These experiments showed that T17N dominant negative Rac inhibited the association of cortactin with N-cadherin compared with wild-type Rac or empty vector controls (Figure 8D: 53 and 24% reduction, respectively).

To verify the biochemical results gathered above, we also examined the functional significance of Rac activation in the translocation of cortactin to sites of nascent N-cadherin ligation using Rat-2 fibroblasts transfected with GFP-tagged Rac constructs. Immunofluorescence analysis of transfected cells stained with cortactin antibodies showed that cortactin recruitment to N-cadherin-mediated intercellular junctions was impaired in cells expressing the T17N Rac construct compared with cells expressing wild-type Rac supporting the obtained biochemical data (Figure 8E).

These data indicate that specific Rho family GTPases are sequentially activated during the maturation of N-cadherin contacts, independent of Fer protein expression or kinase activity, and that transient Rac activation at nascent junctions contributes to cortactin recruitment.

DISCUSSION

Formation and maturation of N-cadherin-dependent intercellular junctions requires a finely tuned interplay between various signaling pathways and cytoskeletal elements. In

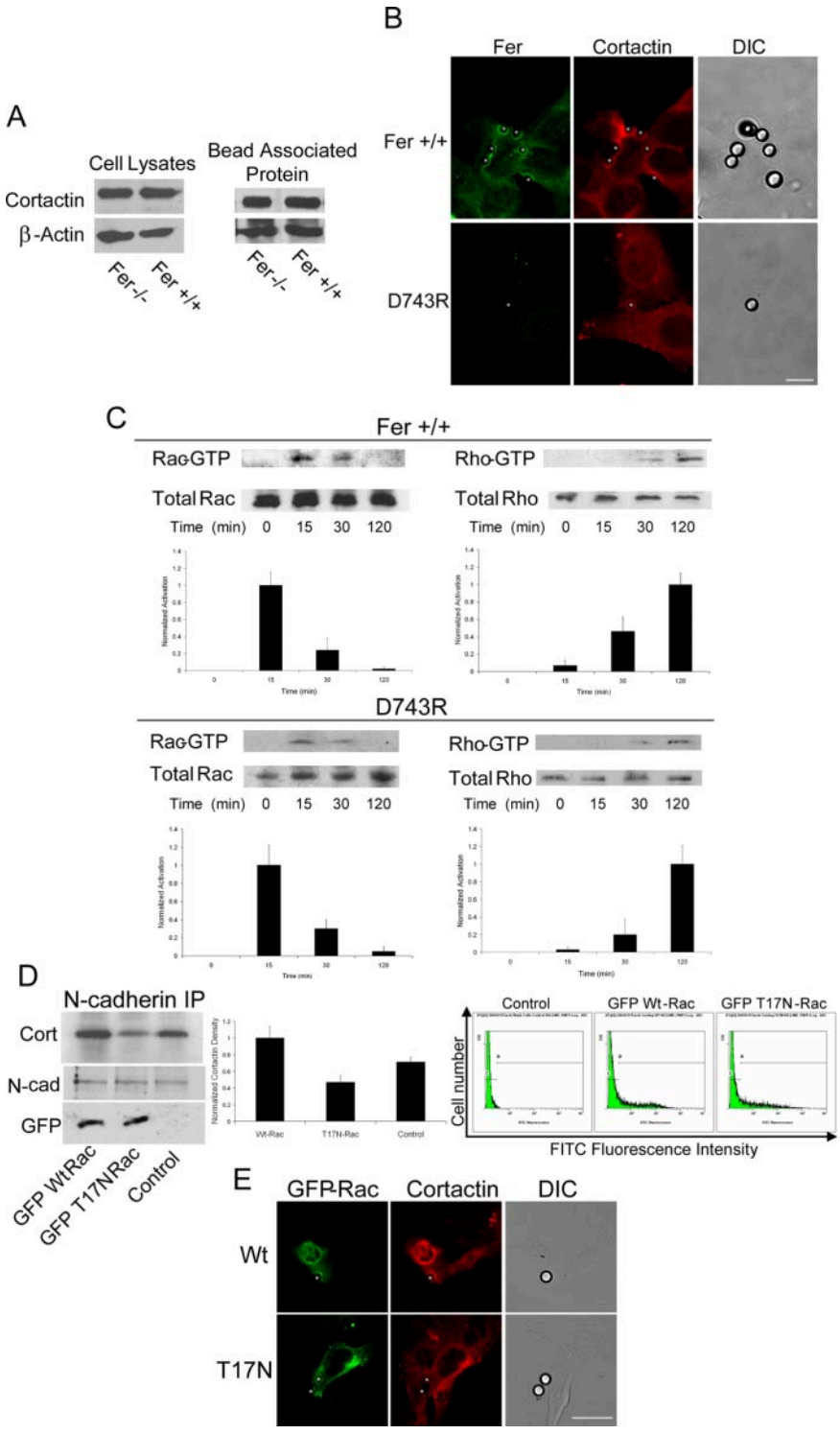

Figure 8. Cortactin recruitment to nascent N-cadherin ligation depends on transient Rac activation but is independent of Fer kinase activity. (A) Equivalent expression of cortactin in whole cell lysates of equal protein concentration in D743R and Fer+/+ fibroblasts. Ncad-Fc-coated magnetic bead pull-off after a 30-min incubation reveals equivalent amount of cortactin recruited to sites of N-cadherin ligation. (B) Immunofluorescence staining of Fer or cortactin in Ncad-Fc bead bound D743R and Fer+/+ cells after 15-min incubation reveal that recruitment of cortactin to sites of N-cadherin ligation is independent of Fer kinase activity. Results shown representative of ~90% of observed bead bound cells {47 and 44 of 50 examined bead bound wild-type and D743R cells, respectively}. (C) N-cadherin engagement induces time-dependent activation of various rho GTPases. Fer+/+ fibroblasts bound to N-cad-Fc-coated 60-mm microbiological plastic dishes were harvested at distinct time points during the first 2 h of attachment. The GTP-bound forms of Rac and Rho were captured by GST-PBD and GST-Rhotekin, respectively. Results are representative of three independent experiments. (D) Recruitment of cortactin to areas of nascent N-cadherin ligation is dependent on locally activated Rac. Sorted Rat-2 cells transfected with wild-type and dominant negative GFP-tagged Rac constructs were attached to Ncad-Fc-coated substrata before lysis and N-cadherin immunoprecipitation. Blots probed for cortactin reveal reduced association of cortactin in T17N-transfected cells when compared with wild-type or controls ($p < 0.05$). Results are representative of three independent experiments. (E) Use of wild-type and negative (T17N) GFP-tagged Rac constructs demonstrates association of both constructs with areas of N-cadherin ligation and dependence of cortactin recruitment to areas of early N-cadherin ligation on Rac activation. Beads were incubated onto cells for 20 min. Results shown are consistent with 75% of bead bound cells {39 and 35 of 50 examined bead-bound Wt-Rac- and T17N-transfected cells, respectively}. Bar, 20 μm

this study we characterized the formation of nascent N-cadherin-dependent adhesions. Specifically, we found that N-cadherin engagement activates Fer kinase, which in turn is responsible for the ensuing transient cortactin tyrosine phosphorylation at intercellular contacts, a process that is critical for adhesion strengthening (El-Sayegh *et al.,* 2004). N-cadherin engagement also initiates Rac activation, a key process in cortactin recruitment to nascent contacts. Fer kinase is important in the early phase of contact formation through its regulation of N-cadherin mobility at intercellular junctions. We suggest that the increased mobility of N-cadherin is necessary for organization of surface expressed N-cadherin, potentially into lateral clusters and for subsequent contact zone extension, resulting in increased adhesion strength (see scheme in Figure 9).

The association between Fer and N-cadherin has been described previously (Kim and Wong, 1995; Rosato *et al.,* 1998; Arregui *et al.,* 2000) and it was recently shown that this

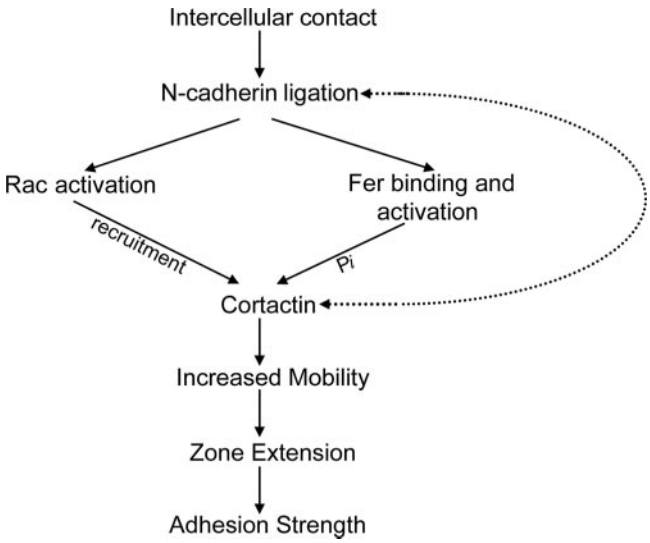

Figure 9. Scheme outlining signaling events downstream of N-cadherin ligation that participate in subsequent enhancement of intercellular adhesion strength.

is mediated by p120 catenin (Xu *et al.*, 2004). Additionally, because both N-cadherin (El Sayegh *et al.*, 2004) and Fer (Kim and Wong, 1998; Kapus *et al.*, 2000; Fan *et al.*, 2004) can associate with cortactin, this spatial organization appears ideal for facilitating specific interactions of an adhesion receptor, an inducible tyrosine kinase and its substrate, an important cytoskeletal regulator. Further, our finding that N-cadherin coprecipitates with nonphosphorylated cortactin in Fer-deficient cells leads to two conclusions. First, cortactin binding to N-cadherin is not mediated by Fer. Second, although tyrosine phosphorylation of cortactin is required for increased adhesion strength, it is not required for cortactin-N-cadherin interactions. The phosphorylation-independent complex formation between these three proteins suggests that the N-cadherin-induced cortactin phosphorylation is catalyzed predominantly by Fer. Indeed, our observation that the N-cadherin-induced phosphorylation of cortactin is markedly inhibited in Fer-deficient D743 cells provides direct experimental evidence for the role of Fer in the process.

To our knowledge this is the first report to indicate that N-cadherin regulates Fer phosphorylation. Specifically, N-cadherin engagement leads to transient tyrosine phosphorylation of Fer, an indicator of enhanced Fer activity (Ben-Dor *et al.*, 1999; Greer, 2002). The mechanism by which N-cadherin ligation stimulates Fer warrants further investigation. Conceivably, conformational changes of N-cadherin may activate Fer, thereby inducing Fer autophosphorylation, or, alternatively, other nonreceptor tyrosine kinases may be recruited to the complex. Notably, p120 catenin, which connects N-cadherin to Fer, is a binding partner of various Src family kinases (Reynolds *et al.*, 1994), and certain members of this kinase family, specifically Fyn and Yes (Kapus *et al.*, 2000; Piedra *et al.*, 2003) have been implicated as upstream activators of Fer.

Although Fer is constitutively associated with N-cadherin, cortactin is recruited to the complex in a phosphorylation-independent manner. Earlier reports proposed that cortactin accumulation at E-cadherin junctions might be mediated by Rac (Helwani *et al.*, 2004). Our study provides strong experimental support to this notion as we found that 1) N-cadherin ligation activates Rac and 2) dominant negative Rac suppresses the association between N-cadherin and cortactin. Although E-cadherin engagement has been unambiguously linked to Rac activation (Noren *et al.*, 2001, 2003; Kovacs *et al.*, 2002a), the regulation of N-cadherin junctions is apparently more complex. Although some studies, in agreement with ours, indicate the importance of Rac in N-cadherin-mediated adhesions (Lambert *et al.*, 2002; Gavard *et al.*, 2004), others do not detect Rac activation (Charrasse *et al.*, 2002) or find that Rac is not required for productive N-cadherin junction formation (Yano *et al.*, 2004). Accordingly, we propose that in addition to cell type-specific differences (Braga *et al.*, 1999), these discrepancies may be explained by two important factors: variations of the type of extracellular matrix (Monier-Gavelle and Duband, 1995, 1997; Levenberg *et al.*, 1998) and the maturation status of contacts. Here we report for the first time that in response to attachment and spreading on substrates coated exclusively with Ncad-Fc ligand, robust Rac activation occurs as an early and transient event that is necessary for the efficient localization of cortactin to the junctions.

Once cortactin is recruited to junctions, it is tyrosine phosphorylated by Fer. Our data show that both the presence of Fer and cortactin, as well as the ensuing tyrosine phosphorylation of Fer and cortactin, are critical factors for adhesion strengthening at the nascent contacts. What is the role of these components in the process? For Fer, a putative mechanism involves Fer-mediated phosphorylation of the phosphatase PTP1B and its subsequent binding to N-cadherin. This step in turn maintains N-cadherin-associated β-catenin in a dephosphorylated state, which is necessary for its stable association with N-cadherin and linkage to the actin cytoskeleton (Xu *et al.*, 2004). Here we propose an additional role for Fer, namely phosphorylation of cortactin.

Cortactin is necessary for normal adhesion assembly, conceivably as an important regulator of the local Arp2/3-dependent actin remodeling at contact sites (Helwani *et al.*, 2004). Although the exact role of cortactin phosphorylation is not well defined, it seems to promote dynamic and rapid actin remodeling, presumably by facilitating the disassembly and subsequent recycling of the cortical actin meshwork (Weed and Parsons, 2001; Fan *et al.*, 2004). Consistent with this view, cells expressing a cortactin mutant lacking three key tyrosine phosphorylation sites show grossly disturbed actin dynamics that manifest as impaired migration and invasion (Li *et al.*, 2001). Importantly, nascent cell adhesions are sites of intensive actin remodeling (Bershadsky, 2004) and cytoskeletal dynamics are a key determinant of the maturation and enhanced strength adhesion receptors (Chu *et al.*, 2004). Several findings reported here support the concept that cortactin phosphorylation is an important regulator of cell contact formation. First, D743R cells, which possess significantly reduced levels of tyrosine phosphorylated cortactin at contacts, also exhibit reduced lateral mobility of N-cadherin at nascent contacts. Second, these cells show significantly reduced clustering subsequent to N-cadherin ligation, and a similar inhibition can be obtained after cortactin down-regulation in wild-type cells. Additionally D743R cells demonstrated reduced adhesion strength in shear wash-off assays and reduced rates of zone extension in spreading assays. Third, we have shown earlier (El-Sayegh *et al.*, 2004) that overexpression of nonphosphorylatable cortactin diminishes adhesion strength in cells expressing wild-type Fer. This report indicates that the presence and kinase activity of Fer is not sufficient for enhancing the strength of adhesions: the accumulation of phospho-cortactin per se is also important. Finally, tyrosine phosphorylation of cortac-

tin is a characteristic of the initial phases of contact formation, coincident with rapid actin remodeling (Chu *et al.*, 2004), whereas it subsides as the contacts mature.

On the basis of these results, we suggest that tyrosine phosphorylation of cortactin, when localized to intercellular junctions, plays an important role in mobilizing N-cadherin at nascent contacts. We consider that this is an important requirement for clustering and enhanced adhesion strength. Indeed, lateral clustering of surface-expressed cadherin molecules is a crucial determinant of adhesive strength, an effect regulated by the cadherin cytoplasmic tail but independent of changes in cadherin-catenin composition (Yap *et al.*, 1997). Conceivably, Fer plays multiple roles in regulating adhesive strength: it may facilitate the process by inducing β-catenin dephosphorylation and by promoting cortactin phosphorylation. Further, our data indicate that β-catenin may not be the only link between cadherins and the actin cytoskeleton: cortactin may also connect cadherins to actin filaments because cortactin binds actin filaments and the Arp2/3 complex (Weed *et al.*, 2000), and associates with E- and N-cadherins. The cortactin-N-cadherin complex may thus represent an additional regulatory locus in linking adhesion molecules to the cytoskeleton.

In summary our results define the N-cadherin-Fer-phospho-cortactin pathway as an important regulatory circuit in which N-cadherin engagement regulates its own clustering, and ultimately, intercellular adhesion strength.

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