Developmental Biology 349 (2011) 363–377

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Modulation of N-cadherin junctions and their role as epicenters of differentiation-specific actin regulation in the developing lens

Michelle Leonard [a], Liping Zhang [a], Ni Zhai [a], Ahmad Cader [a], Yim Chan [a], Roberta B. Nowak [b], Velia M. Fowler [b], A. Sue Menko [a,*]

[a] Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA 19107, USA
[b] Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037, USA

ARTICLE INFO

Article history:
Received for publication 6 July 2010
Revised 5 October 2010
Accepted 9 October 2010
Available online 20 October 2010

Keywords:
N-cadherin
α-catenin
β-catenin
Actin cytoskeleton
Morphogenesis
Lens cell differentiation

ABSTRACT

Extensive elongation of lens fiber cells is a central feature of lens morphogenesis. Our study investigates the role of N-cadherin junctions in this process in vivo. We investigate both the molecular players involved in N-cadherin junctional maturation and the subsequent function of these junctions as epicenters for the assembly of an actin cytoskeleton that drives morphogenesis. We present the first evidence of nascent cadherin junctions in vivo, and show that they are a prominent feature along lateral interfaces of undifferentiated lens epithelial cells. Maturation of these N-cadherin junctions, required for lens cell differentiation, preceded organization of a cortical actin cytoskeleton along the cells' lateral borders, but was linked to recruitment of α-catenin and dephosphorylation of N-cadherin-linked β-catenin. Biochemical analysis revealed differentiation-specific recruitment of actin regulators cortactin and Arp3 to maturing N-cadherin junctions of differentiating cells, linking N-cadherin junctional maturation with actin cytoskeletal assembly during fiber cell elongation. Blocking formation of mature N-cadherin junctions led to reduced association of α-catenin with N-cadherin, prevented organization of actin along lateral borders of differentiating lens fiber cells and blocked their elongation. These studies provide a molecular link between N-cadherin junctions and the organization of an actin cytoskeleton that governs lens fiber cell morphogenesis in vivo.
© 2010 Elsevier Inc. All rights reserved.

Introduction

Cadherin-mediated cell–cell interactions are central to the morphogenetic processes by which differentiated tissue cytoarchitecture is established (Gumbiner, 1996; Larue et al., 1996; Takeichi, 1991; Takeichi, 1995a,b). While it is not yet understood how cadherin junctions drive morphogenesis in vivo, likely pathways can be inferred from in vitro studies. Particularly important among these studies are investigations that link the formation of cadherin junctions with the activation of signaling pathways that direct organization of actin cytoskeletal networks along the cell cortex that, in turn, drive the extension of cadherin junctions along cell–cell interfaces (Vasioukhin et al., 2000; Jamora and Fuchs, 2002; Kovacs et al., 2002a,b; Perez-Moreno et al., 2003; Verma et al., 2004).

Another important element of the role of cadherins in morphogenesis is the regulation of cadherin junction maturation. In vitro studies show that the recruitment of α-catenin to nascent cadherin junctions is a significant component of the mechanism by which cells form close contacts with their near neighbors (Drees et al., 2005; Kobielak and Fuchs, 2004; Bajpai et al., 2008, 2009). α-catenin associates with cadherin complexes through its linkage to either β- or γ-catenin, catenin family proteins that bind directly to the cadherin cytoplasmic domain in a mutually exclusive manner. Decreased tyrosine phosphorylation of β-catenin is believed to be essential for the association of α-catenin with β-catenin (Balsamo et al., 1996) and the dephosphorylation of β-catenin is mediated by phosphatases like SHP-2 (Ukropec et al., 2000). While this mechanism was long thought to bring α-catenin to cadherin complexes for a role as the essential link between cadherin/β-catenin complexes and the actin cytoskeleton, it is now known that α-catenin cannot bind simultaneously to β-catenin and actin (Drees et al., 2005; Yamada et al., 2005). Instead, studies show that recruitment of α-catenin to cadherin complexes plays a crucial role in strengthening intercellular cadherin bonds in a mechanism independent of the actin cytoskeleton (Bajpai et al., 2008, 2009). While α-catenin can be a crucial regulator of a cell's decision to form stable adhesion complexes, it has not yet been investigated whether α-catenin plays a similar role in the maturation of cadherin junctions during tissue morphogenesis in vivo.

In a clever approach in which E-cadherin receptors of cultured cells are engaged by E-cadherin ectodomains coated onto the tissue culture substrate, it was revealed that cadherin junctions can serve as

Abbreviations: EC, central epithelium; EQ, equatorial epithelium; FP, cortical fiber; FC, nuclear fiber; TZ, transition zone.

* Corresponding author. Department of Pathology, Anatomy and Cell, Biology, Thomas Jefferson University, 571 Jefferson Alumni Hall, 1020 Locust Street, Philadelphia, PA 19107, USA. Fax: +1 215 923 3808.
E-mail address: sue.menko@jefferson.edu (A.S. Menko).

0012-1606/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2010.10.009

sites for de novo actin filament assembly through their recruitment and activation of molecules that signal actin nucleation and polymerization, including cortactin and the actin-related protein (Arp)2/3 complex (Helwani et al., 2004; Kovacs et al., 2002b; Kovacs and Yap, 2002). Cortactin is a scaffolding protein believed to link to the cadherin complex by binding to p120 catenin (Boguslavsky et al., 2007). p120 binds directly to the juxtanuclear region of the cadherin cytoplasmic domain, a binding domain distinct from that of β-catenin. The Arp2/3 actin nucleator complex binds directly to cortactin, an association that activates Arp2/3 and stimulates Arp2/3-mediated actin polymerization (Higgs and Pollard, 2001; Uruno et al., 2001; Weaver et al., 2001). While this pathway is described in culture, it is not known if actin regulators such as cortactin and Arp2/3 are recruited to cadherin junctions in vivo to regulate actin assembly for morphogenetic processes required for tissue development.

The embryonic chicken lens is an ideal model in which to study the mechanistic link between the formation of stable cadherin cell–cell junctions and the assembly of an actin cytoskeleton that allows for the rapid extension of cell–cell adhesions necessary for cell and tissue morphogenesis. Development of the lens involves a series of highly coordinated morphogenetic processes that lead to the formation of a greatly ordered structure capable of focusing images, unobstructed, on the retina. At the developmental stage we have studied, embryonic day 10, multiple stages of differentiation are expressed concurrently, providing a snapshot in time of the full spectrum of lens differentiation-specific morphogenesis (diagramed Fig. 1A). This property makes it possible to coordinate biochemical studies of the differentiation stage-specific linkage between cadherin complexes and molecular regulators of morphogenesis with imaging studies that reveal the organization and spatial localization of these molecules in vivo.

In previous studies from our laboratory we show that, in vivo, dramatic reorganization of the actin cytoskeleton is a prominent feature of the differentiation of lens epithelial cells into highly elongated lens fiber cells (Weber and Menko, 2006). As differentiation commences, the predominant actin stress fibers and lamellipodial filaments along the basal surfaces of undifferentiated cells of the lens epithelium are lost and later replaced with an extensive network of cortical actin filaments along the lateral interfaces of young, differentiating lens fiber cells (Weber and Menko, 2006). In studies with primary lens epithelial cell cultures we show that exposing undifferentiated lens epithelial cells to the ROCK inhibitor Y-27632 causes a switch in actin filaments from stress fibers to cortical fibers and, coincidently, the induction of lens differentiation-specific gene expression (Weber and Menko, 2006). These findings directly implicate the active reorganization of the actin cytoskeleton in the process of lens differentiation, while other studies with this same culture system show that formation of stable N-cadherin junctions is required for the formation of differentiated lens structures called lentoids (Ferreira-Cornwell et al., 2000); yet, the relationship between cadherin junction maturation, assembly of a cortical actin cytoskeleton and lens fiber cell morphogenesis remains unknown. In this study we investigate the mechanism by which stabilization of N-cadherin junctions and N-cadherin-directed actin remodeling drives lens fiber cell morphogenesis and establishes lens cytoarchitecture in vivo.

Materials and methods

Immunofluorescence analysis

For immunofluorescence studies of chicken embryo lenses, cryosections were prepared from embryonic day 10 (E10) or E3.5 chick lenses and immunostained as described previously (Walker and Menko, 1999). Briefly, lenses were fixed with 3.7% formaldehyde (methanol for immunolocalization with the N-cadherin and α-catenin at E10) and 20 μm sections were cut using a cryostat. Sections were extracted with 0.25% Triton X-100 prior to immunofluorescence staining. For high resolution analysis of junctional proteins lens epithelial explants were isolated from the E10 lens. This was accomplished by removal of the fiber cell mass from the lens proper through the posterior aspects of the lens, leaving behind the entire lens epithelium attached to its endogenous basement membrane, the lens capsule. The resultant epithelial explants, which had been fixed with 3.7% formaldehyde, were pinned to a culture dish, cell side up, for immunolocalization analysis. The explants consisted principally of an epithelial sheet; the undifferentiated epithelial cells in the center of the explant, which had lined the anterior surface of the lens, were surrounded by the lens epithelial cells of the equatorial zone where differentiation initiation occurs and, at their outermost edges, were in turn surrounded by cells of the lens transition zone where lens fiber cell elongation/maturation begins. For immunostaining, samples were incubated sequentially with primary antibody (N-cadherin (Zymed), β-catenin (BD Transduction, San Jose, CA), α-catenin (Sigma, St. Louis, MO), or cortactin (Upstate, Lake Placid, NY)) and fluorescence-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). F-actin was localized in formaldehyde fixed sections with Alexa448-conjugated phalloidin (Invitrogen-Molecular Probes Eugene, OR). Imaging of immunostained cryosections and epithelial explants was performed by confocal microscopy analysis using a Zeiss LSM510 META confocal microscope. Z-stacks were collected and analyzed; the data presented represents a single optical plane in the Z-stack. Within each study all images were acquired with the same settings.

For immunofluorescence staining of mouse lenses, eyes from 1 month-old animals were dissected in PBS, cut open at the posterior pole, fixed in 1% (for N-cadherin) or 4% (for Arp3) paraformaldehyde in PBS for 4 h at 4 °C, followed by incubation overnight in 30% sucrose in PBS and embedding in OCT. Blocks were sectioned and immunostained as described in Nowak et al. (2009). Rhodamine-phalloidin (1:200 of 200 units/ml, Molecular Probes) was used to label F-actin. Primary antibodies were a mouse monoclonal against Arp3 (2.5 μg/ml) (BD Transduction Labs) and a rat monoclonal against N-cadherin (1:10) (a gift from Dietmar Vestweber, Max-Planck-Institute of Molecular Biomedicine, Germany). Secondary antibodies were Alexa Fluor-647 goat-anti-mouse and Alexa Fluor-350 goat-anti-rat (Molecular Probes). Images were acquired at RT using a Bio-Rad 2100 Radiance Laser-Scan Confocal mounted on a Nikon Microscope using a 100×/1.4 n.a. objective lens (Zoom 1).

Microdissection of embryonic lenses

Lenses were isolated from E10 chicken eggs (CBT Farms, Chestertown, MD) and microdissected as previously described to yield four distinct regions of differentiation: the central anterior epithelium (EC), the equatorial epithelium (EQ), cortical fiber cells (FP), and the central fiber zone (FC), as described previously (Walker and Menko, 1999).

Protein extraction, standard immunoprecipitation and immunoblotting

Tissue samples were extracted in Triton/Octylglucoside (OG) buffer (44.4 mM n-Octyl β-D glucopyranoside, 1% Triton X-100, 100 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA, 10 mM imidazole, pH 7.4). The extraction buffer contained 1 mM sodium vanadate, 0.2 mM H_2O_2, and Protease Inhibitor Cocktail (Sigma). Protein concentrations were quantified using the BCA assay (Pierce, Rockford, IL). For direct immunoblot analysis 15 μg of protein extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) on precast 4–12% Tris–Glycine gels (Novex, San Diego, CA). For immunoprecipitation studies, the entire sample from each microdissected compartment from a set of 100 lenses was used. This best represented similar levels of N-cadherin expression across the microdissected fractions, accounting for the fact that crystallins comprise increasingly larger proportions of the cellular protein as the lens cells differentiate. For standard immunoprecipitations, samples were incubated at 4 °C sequentially with primary antibody and either protein G (Sigma, St. Louis, MO) or TrueBlot Immunoprecipitation beads (eBioscience) and the immunoprecipitates

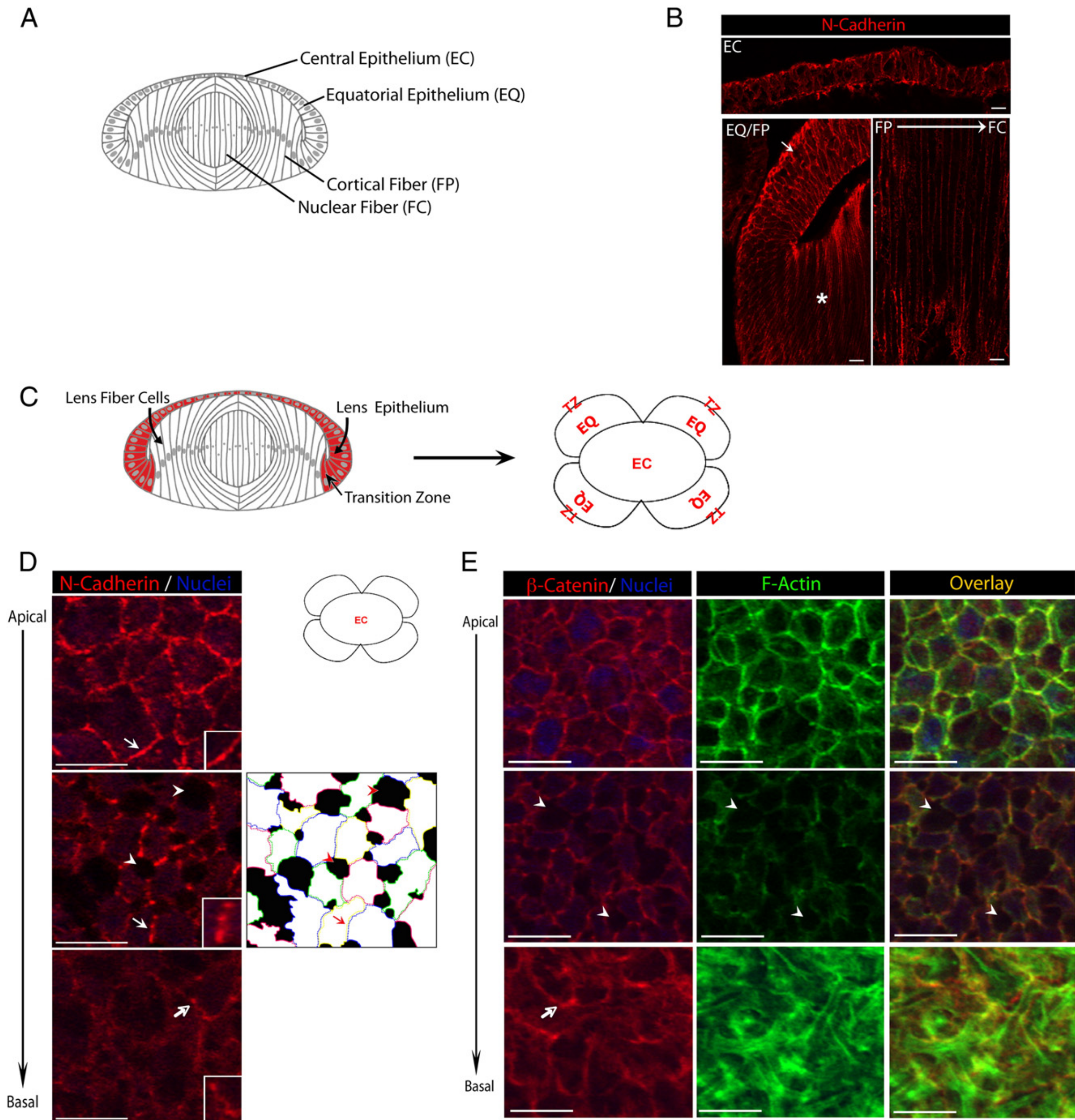

Fig. 1. N-cadherin junctional complexes in the embryonic lens. (A) Diagram of the E10 chick lens demonstrating multiple zones of differentiation: (EC) central epithelium, undifferentiated lens epithelial cells; (EQ) equatorial epithelium, differentiation initiation; (FP) cortical fiber cells, fiber cell elongation/morphogenetic differentiation; (FC) nuclear fiber cells, maturation. (B) Immunolocalization of N-cadherin in an E10 chick lens section. Maturation of N-cadherin junctions along lateral cell–cell borders was observed in the EQ and FP zones (in EQ/FP panel, EQ is denoted by arrow, FP by asterisk). The complexity of N-cadherin junctional organization as a function of lens cell differentiation was examined in detail in isolated lens epithelial explants (diagrammed in C) that contain EC, EQ and transition (TZ) zones, TZ where fiber cell elongation begins. The explants were imaged by confocal microscopy in the EC zone (D,E) following immunostaining for N-cadherin (D), or for β-catenin in samples double-stained with fluorescent phalloidin to visualize F-actin (E). Z-stacks were collected from apical to basal aspects of these undifferentiated lens epithelial cells and the data presented is from selected optical planes within a stack. Results are representative of at least 3 independent experiments. D: (Top panel) Mature N-cadherin junctions were detected at cell–cell borders only in these apical-most aspects of the undifferentiated epithelium (arrow and inset). (Middle panel) Lateral cell interfaces contained only nascent N-cadherin junctions that were localized to limited regions of contact between lamellipodial and filopodial extensions from neighboring cells (puncta, arrow and inset); many gaps were observed between cells (arrowheads, diagrammed at right). (Bottom panel) At the basal cell surface N-cadherin localized to the edges of lamellipodial extensions (arrow and inset). E: (Top panels) Both β-catenin and F-actin were highly localized to the apical cell cortex. (Middle panels) Gaps (arrowheads) were observed along the lateral interfaces of these cells as diagramed in 1D, but in areas of cell–cell contact β-catenin appeared linear. Little organized actin was detected along these lateral borders. (Bottom panels) β-catenin is present at the edges of lamellipodial extensions (arrow) and F-actin was organized in stress fibers and in lamellipodia along the cells' basal cell surfaces. Bar, 10 μm.

subjected to SDS/PAGE as described previously (Walker et al., 2002). Proteins were electrophoretically transferred from the gels onto Immobilon-P membranes (Millipore, Bedford, MA) and detected using standard Western Blot techniques (Walker et al., 2002). Antibodies used included N-cadherin (BD Transduction, St. Louis, MO), β-catenin, γ-catenin and Arp3 (BD Transduction, St. Louis, MO), α-catenin, p120 catenin and β-actin (Sigma, St. Louis, MO), anti-phosphotyrosine 4G10 (Upstate, Lake Placid, NY), SH-PTP2 (Santa Cruz, Santa Cruz, CA), and cortactin (Upstate, Lake Placid, NY). Secondary antibodies conjugated to horseradish peroxidase (Jackson Laboratories, West Grove, PA) were

detected using ECL reagent from Amersham Biosciences (Piscataway, NJ). Immunoblots were scanned and densitometric analysis was performed using Kodak 1D software (Eastman Kodak Company, Rochester, NY). To standardize these results quantitative analysis was performed. For each protein analyzed, the densitometry results first were normalized to one differentiation-specific zone, and then the ratio of each co-precipitated protein to the original immunoprecipitated protein was calculated for each fraction.

Determination of tyrosine phosphorylation of β-catenin in N-cadherin complexes

For analysis of the state of tyrosine phosphorylation of β-catenin that is linked to N-cadherin complexes the samples were immunoprecipitated first for N-cadherin as described above, the washed pellet resuspended in 1% SDS, 20 mM Tris–HCl, pH 7.6, 100 mM NaCl, 1 mM Na_3VO_4, and 0.2 mM H_2O_2 and boiled for 3 min. The now separated N-cadherin complex proteins were subjected to immunoprecipitation as described above with a β-catenin-specific antibody, followed by immunoblot analysis with the phosphotyrosine antibody 4G10. There is no reassembly of cadherin complex proteins in the SDS buffer.

Double immunoprecipitation for identification of more than two components of a cadherin complex

Standard immunoprecipitation protocols only identify bivalent associations between proteins in a receptor complex. A novel sequential immunoprecipitation protocol was developed by our laboratory in order to identify multiple members of a single receptor complex (Leonard et al., 2008). This new technique employs the ProFound assay (Pierce, Rockford, IL) to purify intact N-cadherin receptor complexes without the IgG that is used in their isolation. The isolated N-cadherin complexes were immunoprecipitated with an antibody to a second component of the complex, β-catenin or γ-catenin, and then immunoblotted for a third protein in the receptor complex, α-catenin, cortactin or Arp3. By performing this study on microdissected fractions of the E10 chick lens it was possible to place the assembly of a particular N-cadherin complex in a specific time and place in lens development.

Ex vivo explant cultures

Epithelial explants were prepared from embryonic day 10 chicken lenses as described above, pinned cell side up in a tissue culture dish and grown in Media199 (Invitrogen) with 10% fetal bovine serum (Invitrogen). N-cadherin junction formation was blocked by growing the explant cultures in the presence of the function-blocking antibody NCD-2 (Zymed).

Results

Differentiation-state specific organization of N-cadherin junctions

Previous studies from our laboratory with a primary lens epithelial cell culture system show that nascent N-cadherin junctions (puncta) zip up to form mature, linear junctions as lens cells differentiate in vitro and that this maturation of N-cadherin junctions is required for the differentiation of lens epithelial cells in vitro (Ferreira-Cornwell et al., 2000). Now, using confocal microscopy image analysis, we investigated the state of organization of N-cadherin junctions in vivo and how these junctions change with lens cell differentiation state, both temporally and spatially. Immunofluorescence analysis performed on E10 lens sections showed that N-cadherin junctions were organized as discontinuous puncta along the lateral interfaces of undifferentiated lens epithelial cells (EC, Fig. 1B), consistent with their presence in nascent junctions, with a greater concentration of N-cadherin junctions at the apical and basal aspects of these cells. Linear aligned N-cadherin junctions, typical of mature junctions, first appeared along lateral borders of lens cells in the posterior regions of the equatorial zone (EQ), the region of the lens where differentiation is initiated (Fig. 1B, EQ). Mature N-cadherin junctions were present along the lateral interfaces of lens fiber cells throughout their morphogenetic differentiation in the cortical fiber zone (FP) of the lens (Fig. 1B, FP), the same region where a cortical actin cytoskeleton is assembled (Weber and Menko, 2006). The observation that N-cadherin junctions mature in the EQ zone prior to the organization of a cortical actin cytoskeleton places these junctions in the appropriate time and place to act as a nucleation sites for assembly of the actin cytoskeleton in differentiating lens fiber cells.

Approach for a detailed analysis of N-cadherin junctional organization in the embryonic lens

In order to perform a more detailed analysis of the complexity of N-cadherin junctions along the lateral borders of lens epithelial cells in vivo and understand how these junctions change their organization both as differentiation is initiated in the EQ zone and as elongation commences in the transition zone (TZ), the region between lens epithelial and fiber cells, lens epithelial cell sheets were isolated intact, still attached to their endogenous basement membrane. This region of lens tissue, which contained the entire lens epithelium with the EC zone in the center surrounded by the epithelial cells of the EQ zone and then by the newly differentiating fiber cells in the TZ (Fig. 1C), was pinned to a supportive platform cell side up. The cells were immunostained with antibodies to the cadherin complex proteins N-cadherin, β-catenin, and α-catenin, and imaged by confocal microscopy (Figs. 1–3). Multiple optical planes were collected from apical to basal aspects of the cells as Z-stacks. This analysis revealed a previously unrecognized complexity to the cadherin junctions of lens epithelial cells, in addition to the expected changes in cadherin junctions related to lens cell differentiation state observed in the lens section studies.

Complexity of N-cadherin junctions in undifferentiated lens epithelial cells

We were surprised to find as many as three separate regions of N-cadherin junctions along the lateral cell–cell interfaces of undifferentiated lens epithelial cells, each with a distinctive type of organization (EC, Fig. 1D, modeled in Fig. 10). Along the apicolateral aspects of these cells was a continuous band of N-cadherin/β-catenin junctions that co-localized with the cortical actin cytoskeleton (Figs. 1D and E). In these junctions α-catenin was most noticeable for its absence, with only low levels of linear staining detected (Fig. 3A). The position of these apicolateral N-cadherin junctions provided the first evidence that a distinct subpopulation of N-cadherin junctions in lens epithelia may organize the apical junctions that hold the lens epithelium together as a sheet. Another discrete pattern of organization of N-cadherin junctions was discovered in the basal aspects of the lens epithelial cells where they extend actin-rich lamellipodia along the basement membrane capsule. N-cadherin (Fig. 1D), β-catenin (Fig. 1E) and α-catenin (Fig. 3A) all localized to the tips of these lamellipodia extensions (arrows in bottom panels of Figs. 1D, E and 3A) possibly for a role in directing movement of the lens epithelial cells on the capsule. Most novel; however, was the third region of cadherin junctional organization that was identified in these studies of undifferentiated epithelial cells. These junctions were associated with newly discovered membrane protrusions that extended across the large spaces between the opposing lateral membranes of neighboring epithelial cells. Nascent N-cadherin junctions (Fig. 1D, arrow, middle panel) with no detectable α-catenin (Fig. 3A, middle panel) were present at the sites of contact between these lamellipodial-like extensions, often appearing as puncta. These junctions were the only regions of cell–cell contact along the mid-lateral aspects of undifferentiated lens epithelial cells. We believe that this is the first report of

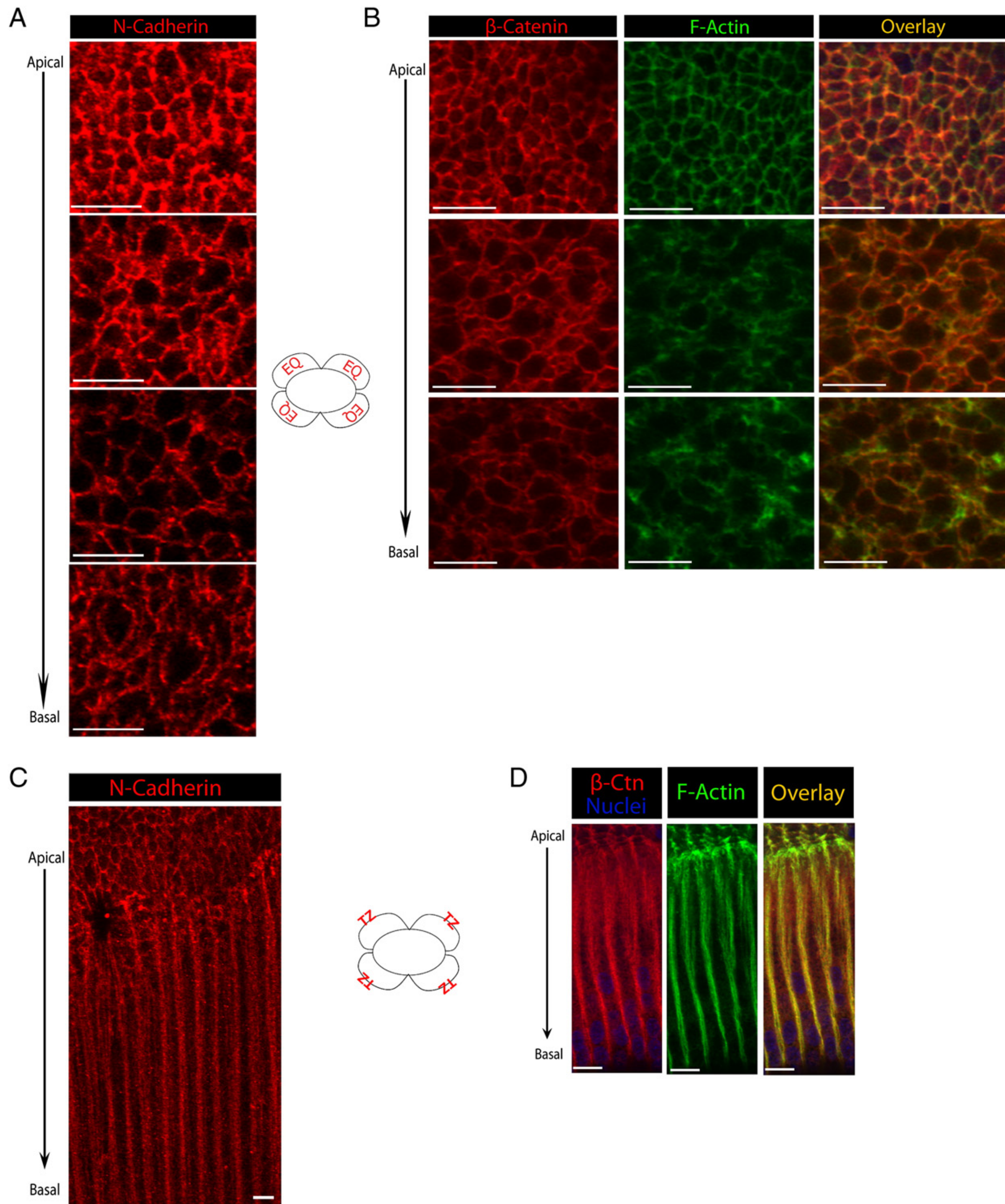

Fig. 2. Maturation of N-cadherin junctional complexes along the cells' lateral borders is coincident with lens differentiation initiation and elongation. Lens epithelial explants were examined by confocal microscopy following immunostaining for N-cadherin (A,C) or β-catenin co-stained for F-actin with fluorescent phalloidin (B,D). Explants were imaged in the EQ zone (A,B) where lens epithelial differentiation is initiated, and in the TZ region (C,D) where lens fiber cell elongation begins. In the EQ zone N-cadherin junctions zip up lateral cell interfaces as evidenced by prominent, apical to basal staining for mature N-cadherin junctions (A) and for the cadherin complex protein β-catenin (B). As in undifferentiated cells; however, F-actin localization in the EQ zone was great at the apical cortex but only a minimal level of F-actin organization was detected at the lateral cell–cell borders of these cells. In contrast, in the transition zone (TZ — C,D), N-cadherin (C), β-catenin (D) and F-actin (D) all were highly localized along the lengths of the cell–cell borders of these elongating lens fiber cells. For each molecule observed images were acquired with the same settings as in Fig. 1 and results are representative of at least 3 independent studies. Bar, 10 µm.

nascent cadherin junctions in vivo. β-catenin was present in these nascent junctions (Fig. 1E, middle panel), but its distribution was more linear than that of N-cadherin, likely reflecting the co-expression of N-cadherin and E-cadherin in lens epithelial cells (Pontoriero et al., 2009). Our studies focus on N-cadherin junctions as E-cadherin disappears as lens cells differentiate (Pontoriero et al., 2009; Xu et al., 2002).

α-catenin associates with cell–cell interfaces of lens epithelial cells in the equatorial zone as N-cadherin junctions mature

As lens differentiation is initiated in the equatorial zone, the epithelial cells become compact and columnar (note the smaller cell diameter in Figs. 2A and B compared to cells in Figs. 1D and E); a

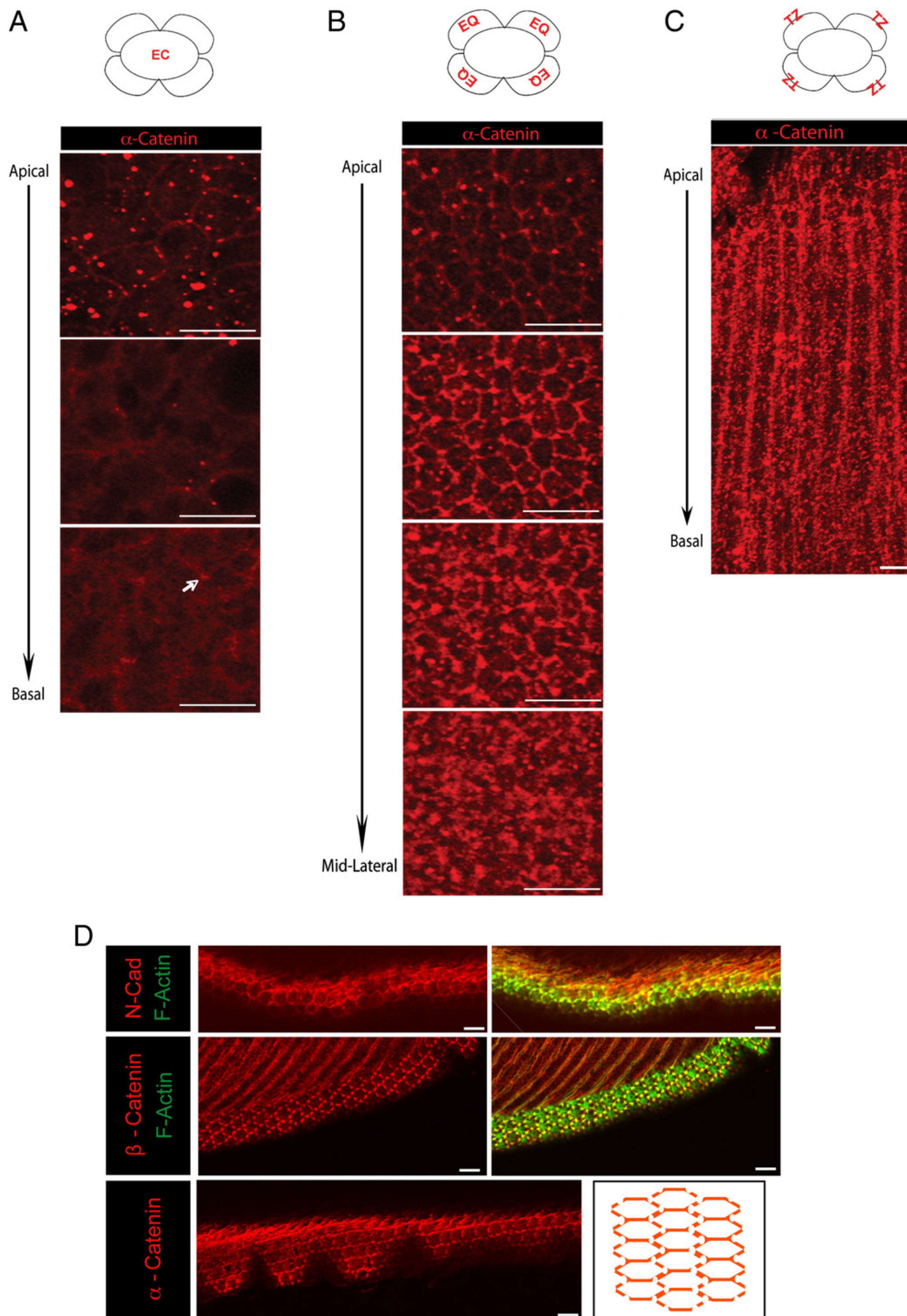

Fig. 3. Localization of α-catenin to cell–cell interfaces is coincident with the maturation of N-cadherin junctions and the initiation of differentiation. (A–C) Immunolocalization of α-catenin in epithelial explants viewed as individual optical sections from Z-stacks collected by confocal microscopy showed dramatic changes in the distribution of α-catenin between the EC (A), EQ (B) and TZ (C) zones. High levels of α-catenin at lateral membranes first were detected in the EQ zone (B). The images in B represent the four apical-most optical sections. In the TZ, linear aligned puncta of α-catenin were observed all along cell–cell interfaces of these elongating cells. All images in A–C were acquired with the same settings and the results are representative of at least 3 independent studies; bar, 10 μm. (D) Cross-sections of the cortical fiber zone (FP) near the posterior region of the E10 lens were viewed by confocal microscopy following immunostaining for N-cadherin, β-catenin and α-catenin; N-cadherin and β-catenin sections were co-stained for F-actin. Both β-catenin and α-catenin localized to lateral cell–cell interfaces, but not to cell vertices, coincident with F-actin, as modeled in the diagram in D. Images are representative of 3 independent studies; bar, 10 μm.

morphogenetic change that we now report is coordinate with maturation of N-cadherin junctions along their lateral domains (Fig. 2A). While N-cadherin/β-catenin junctions remain a prominent feature at the apicolateral borders of these cells, now these junctions were extended as a continuous band all along the newly aligned cell–cell interfaces (Figs. 2A and B). Consistent with a role for these maturing N-cadherin junctions in zipping up the cells' lateral membranes, spaces were no longer detected between the lens cells of the equatorial epithelium (modeled in Fig. 10). Also in these cells, N-cadherin junctions that had been present in lamellipodial

extensions along the basement membrane were replaced with linearly aligned N-cadherin junctions at these now closely apposed lateral membranes (Fig. 2A, bottom panel). The most striking molecular difference between the nascent junctions of undifferentiated lens epithelial cells and these mature N-cadherin junctions in the EQ zone that had zipped up the lateral cell interfaces was the localization of α-catenin along the cell–cell borders (Fig. 3B, modeled in Fig. 10). The association of α-catenin with these lens cells' lateral membranes was greatest in the top few microns of the cells. The distribution of α-catenin was linear along the cells' apicolateral interfaces and punctate along the remaining areas of cell–cell contact. In mid-lateral regions of these lens cells there also was significant cytoplasmic staining for α-catenin, a finding supported by biochemical studies that showed the presence of a pool of Triton X-100-soluble α-catenin in cells of the lens epithelium (data not shown). α-catenin localization to cell–cell borders becomes more diffuse and less intense at lateral cell interfaces near the basal surfaces of these lens cells (data not shown). Interestingly, filamentous actin did not follow the same pattern in either intensity or distribution as N-cadherin, β-catenin, or α-catenin, and was mostly concentrated in the apical and basal domains of the cells with only limited organization of actin filaments along lateral cell interfaces (Fig. 2B). This finding indicates that the recruitment of α-catenin to N-cadherin junctions preceded the assembly of a cortical actin cytoskeleton along lateral borders of neighboring lens cells.

Actin filaments first co-localize with lateral N-cadherin junctions in elongating fiber cells

At the edges of the explanted epithelium is the transition zone — TZ — a narrow region of elongated cells oriented at almost a right angle to the epithelium proper. These are the cells that turn the corner below the posterior equatorial epithelium just before being added as the outermost differentiating fiber cells in the cortical zone (modeled in Fig. 1C). The lateral cell–cell borders of these transitional cells were stained intensely for N-cadherin (Fig. 2C), β-catenin (Fig. 2D), α-catenin (Fig. 3C), and F-actin (Fig. 2D). It was in this region of the differentiating lens that the localization and relative intensity of N-cadherin, β-catenin, α-catenin, and actin filaments became coincident and linearly aligned; although α-catenin staining still appeared somewhat punctate (modeled in Fig. 10).

In the cortical fiber (FP) region proper the differentiating fiber cells are arranged with precise hexagonal packing (see model Fig. 3D). The distribution of N-cadherin, β-catenin and α-catenin along the cell–cell borders of fiber cells in this FP zone was examined in cross-sections cut near the posterior aspect of the lens. Confocal microscopy analysis of these immunostained sections showed that N-cadherin was localized all along the cell–cell interfaces of the hexagonally packed fiber cells (Fig. 3D). While β-catenin also was present along the lateral interfaces of these cortical fiber cells (Fig. 3D), it was mostly absent from the cell vertices, a region our previous studies show is rich in γ-catenin (Leonard et al., 2008). The pattern of α-catenin localization (Fig. 3D) was similar to that of β-catenin, staining intensely along lateral cell–cell interfaces but absent from cell vertices, a distribution also highly coincident with that of F-actin in these differentiating fiber cells (Fig. 3D).

α-catenin recruitment to lateral interfaces as lens cells form close cell–cell contacts in vivo reflects its association with N-cadherin complexes

Our immunolocalization studies demonstrated that assembly of mature N-cadherin junctions along cell–cell interfaces of lens epithelial cells in the equatorial zone occurred as α-catenin became associated with these cell membranes and preceded the organization of a cortical actin cytoskeleton. We performed co-immunoprecipitation analyses to investigate whether the localization of α-catenin to lateral cell interfaces in the EQ zone reflected its linkage to N-cadherin junctional complexes, an association reported to strengthen interactions between apposing cadherin receptors (Bajpai et al., 2008, 2009). For these studies E10 lenses were microdissected into four differentiation-specific zones: 1) the undifferentiated epithelial cells of the EC zone, 2) the EQ zone where differentiation is initiated, 3) the zone of fiber cell morphogenesis (FP) and 4) the zone of fiber cell maturation (FC) (Fig. 4A). N-cadherin was immunoprecipitated from extracts of each of these fractions and the immunoprecipitates blotted for both β- and α-catenins (Fig. 4B). As we reported previously, the level of association of β-catenin with N-cadherin complexes was unchanged regardless of the differentiation state of the cells (Leonard et al., 2008). In contrast, the association of α-catenin with N-cadherin junctional complexes was differentiation-state specific. Paralleling the immunofluorescence results, there was only a low level association of α-catenin with N-cadherin junctions in the EC zone, while in the EQ zone, where close cell–cell contacts are established, α-catenin was highly recruited to N-cadherin junctional complexes (Fig. 4B). α-catenin also was highly associated with N-cadherin complexes of cells in the FP zone (Fig. 4B), where establishment of new, stable N-cadherin junctions accompanies the elongation of these cells. The assembly of mature N-cadherin junctions in the EQ zone may be a prerequisite to recruiting actin regulators to cell–cell junctions to drive convergent extension of the differentiating lens fiber cells.

Since α-catenin can associate with both cadherin/β-catenin and cadherin/γ-catenin complexes (Hinck et al., 1994; Huber et al., 1997; Kemler, 1993), but only cadherin/β-catenin complexes link exclusively to the actin cytoskeleton, we examined the differentiation-state specific association of α-catenin with N-cadherin/β-catenin complexes. For this study intact N-cadherin complexes were isolated from extracts of each differentiation region of the E10 lens using an N-cadherin antibody-linked column (Leonard et al., 2008). These complexes then were subjected to a sequential immunoprecipitation with antibody to β-catenin, isolating N-cadherin/β-complexes, which then were analyzed by immunoblot for linkage of α-catenin. While there was little association of α-catenin with N-cadherin/β-catenin complexes in undifferentiated epithelial cells, α-catenin was highly recruited to N-cadherin/β-catenin complexes in both the equatorial zone, EQ, and in the cortical fiber zone, FP, (Fig. 4C). These findings support a role for α-catenin in the maturation of N-cadherin/β-catenin junctions both as lens fiber cells begin their differentiation and as they undergo morphogenesis to achieve their highly elongated phenotype. α-catenin dropped in association with N-cadherin/β-catenin junctions in the FC zone (Fig. 4C), a region that contains the most mature fiber cells, but not in direct N-cadherin immunoprecipitations from this region (Fig. 4B), likely reflecting the association of α-catenin with N-cadherin/γ-catenin junctions at later stages of fiber cell differentiation.

Decreased tyrosine phosphorylation of β-catenin as lens cells differentiate linked to recruitment of α-catenin to N-cadherin junctions

Tyrosine phosphorylation of β-catenin, as occurs in response to different stimuli such as growth factors, has been determined to negatively regulate cadherin junction adhesive strength by blocking recruitment of α-catenin to the cadherin complex (Daniel and Reynolds, 1997; Lilien et al., 2002; Ozawa and Kemler, 1998; Roura et al., 1999). Therefore, we examined whether there were differentiation-specific changes in the tyrosine phosphorylation of β-catenin linked to N-cadherin complexes in vivo that could account for the recruitment of α-catenin to N-cadherin junctions. For these studies N-cadherin was immunoprecipitated from extracts of the four differentiation-specific zones of the embryonic lens, EC, EQ, FP, and FC. The isolated N-cadherin complexes were boiled in a 1% SDS buffer to dissociate the complex components and then immunoprecipitated with antibodies to β-catenin, making it possible to isolate β-catenin from proteins in the N-cadherin complex. Immunoblotting of the β-catenin immunoprecipitates with the

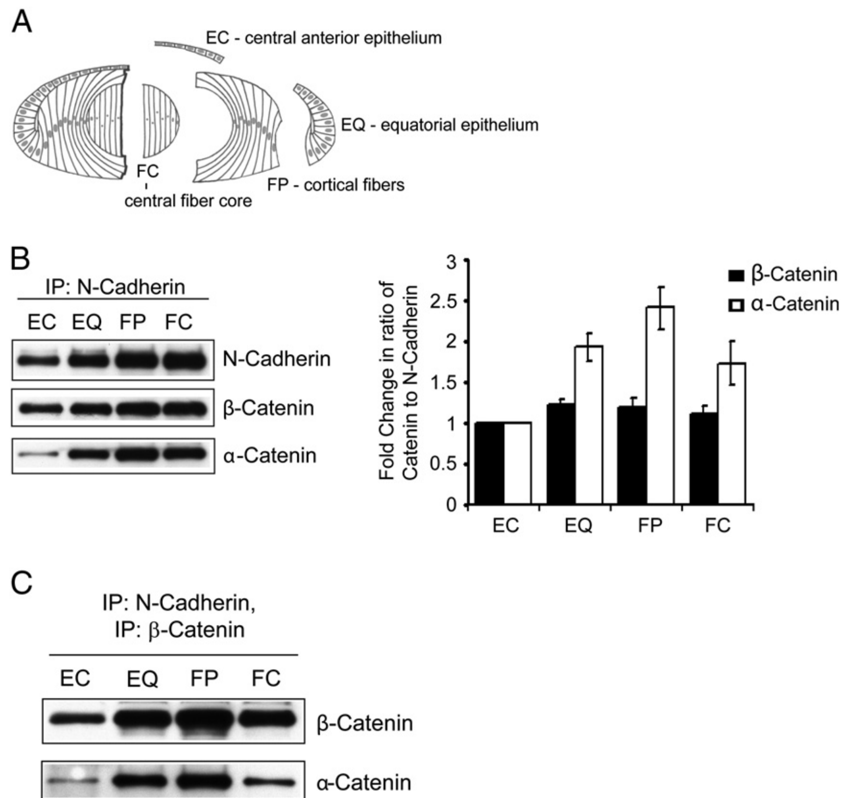

Fig. 4. Association of α-catenin with N-cadherin complexes is coincident with the maturation of N-cadherin junctions. (A) Model of E10 chick lens microdissection to yield the four regions of differentiation: EC, EQ, FP, and FC. (B) Differentiation-state specific recruitment of α-catenin to N-cadherin junctions determined by co-immunoprecipitation analysis; immunoprecipitate for N-cadherin followed by Western Blotting for α-catenin and β-catenin. Densitometric analyses are represented as the ratio of β- or α-catenin to N-cadherin, normalized to EC. There was a great increase in association of α-catenin with N-cadherin junctional complexes in the EQ zone of differentiation initiation, which was maintained throughout lens fiber cell differentiation with no change in the association of β-catenin with N-cadherin. (C) Double immunoprecipitation analysis was used to examine the association of α-catenin with N-cadherin/β-catenin complexes. N-cadherin complexes were isolated with an antibody column and then subsequently immunoprecipitated for β-catenin, thereby isolating N-cadherin/β-catenin complexes. Association of α-catenin with this complex was determined by Western Blot analysis. The results showed that α-catenin was highly recruited to N-cadherin/β-catenin junctions in the EQ zone, coordinated with the zipping up of N-cadherin junctions and remained highly associated with N-cadherin/β-catenin junctions in the differentiating cortical fiber cells of the FP zone.

4G10 phosphotyrosine antibody then revealed the level of tyrosine phosphorylation of N-cadherin-linked β-catenin.

Tyrosine phosphorylation of β-catenin in N-cadherin complexes was very high in undifferentiated cells of the EC region in vivo (Fig. 5A), consistent with both the high levels of nascent N-cadherin junctions (Fig. 1D) and the low level of α-catenin associated with N-cadherin complexes in the EC zone (Figs. 4B and C). There was a clear trend of dephosphorylation of N-cadherin-linked β-catenin as lens cells differentiated in vivo (Fig. 5A). However, in the EQ zone the level of β-catenin phosphorylation proved to be variable and, therefore, difficult to interpret. We suspect that the reason for this variability is the dynamic nature of the EQ zone where lens cells initiate their differentiation, and across which there is a range in the degree of junctional maturation (Fig. 1B). In the adjacent differentiating cortical fiber cells, tyrosine phosphorylation of β-catenin was dramatically reduced, and tyrosine phosphorylation of β-catenin in N-cadherin complexes remained low throughout the remainder of lens fiber cell differentiation. While these differentiation-specific modifications of β-catenin were consistent with a mechanism that permits its association with α-catenin, they did not affect association between β-catenin and N-cadherin (Fig. 4B).

SHP-2 phosphatase recruited to N-cadherin junctions as lens cells initiate differentiation

The dephosphorylation of β-catenin in maturing cadherin junctions is accomplished primarily through the action of protein tyrosine phosphatases (Balsamo et al., 1996; Wadham et al., 2003), such as SH2 domain-containing phosphatase-2 (SHP-2). In the cadherin complex SHP-2 associates directly and uniquely with β-catenin (Ukropec et al., 2000). Immunoblotting of microdissected E10 lens fractions showed that SHP-2 was expressed highly at all stages of differentiation in the embryonic lens (Fig. 5B), but co-immunoprecipitation analysis showed that SHP-2 association with N-cadherin junctions was primarily limited to cells in the EQ and FP zones differentiation (Fig. 5B). The increased association of SHP-2 with N-cadherin during lens cell differentiation corresponded with decreased tyrosine phosphorylation of N-cadherin-linked β-catenin (Fig. 5A), and paralleled the association of α-catenin with N-cadherin/β-catenin complexes (Fig. 4C). This data suggested that SHP-2 was a positive regulator of N-cadherin junctional maturation in the developing lens.

The actin regulator cortactin is highly recruited to N-cadherin junctions during the period of fiber cell elongation

The remainder of these studies investigated whether the morphogenetic differentiation of lens fiber cells involved cadherin-directed organization of the actin cytoskeleton. Studies in vitro show that the actin regulator cortactin is an important intermediate in cadherin-directed actin polymerization (Helwani et al., 2004). Embryonic lens cells expressed this scaffolding protein at every stage of differentiation and morphogenesis, but most highly in cells of the lens epithelium (Fig. 6A). To investigate whether cortactin could be a downstream effector of N-cadherin-directed actin polymerization in vivo, we performed co-immunoprecipitation analysis. For these studies, N-cadherin was immunoprecipitated from extracts of each

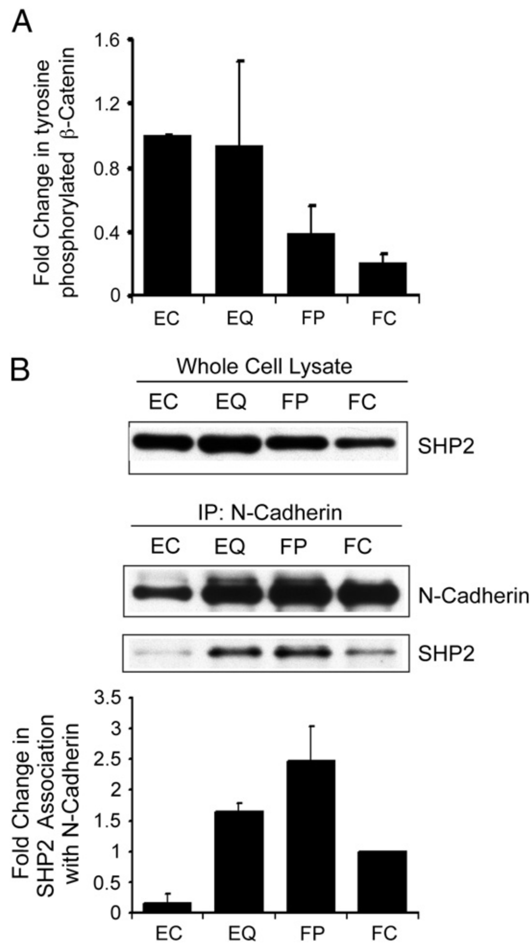

Fig. 5. Decreased tyrosine phosphorylation of N-cadherin associated β-catenin with lens cell differentiation may be regulated by SHP-2. (A) β-catenin was immunoprecipitated from dissociated N-cadherin complexes and Western Blotted for phosphotyrosine (4G10) and β-catenin. Following densitometry analysis the results were plotted as the fold change of the ratio of tyrosine phosphorylated β-catenin/β-catenin in N-cadherin complexes, relative to EC. Tyrosine phosphorylation of β-catenin in N-cadherin junctions dramatically decreased with fiber cell differentiation. (B) Western Blot analysis of whole cell lysates showed that SHP-2 was expressed at all stages of lens differentiation. The recruitment of SHP-2 to N-cadherin junctions and the differentiation-state specificity of this interaction were determined by immunoprecipitation for N-cadherin followed by Western Blot for SHP-2. Densitometric analysis was plotted as the fold change in SHP-2 recruitment to N-cadherin with differentiation state, relative to FC, revealing that the SHP-2 phosphatase associated with N-cadherin complexes coincident with dephosphorylation of β-catenin. Results are representative of at least 3 independent studies.

differentiation-specific region of the lens (EC, EQ, FP and FC) and the immunoprecipitates were immunoblotted with antibody to cortactin. Little cortactin was associated with N-cadherin complexes in the undifferentiated lens cells of the EC zone (Fig. 6A), even though cortactin was highly expressed by these cells. Therefore, the presence of cortactin in lens epithelial cells likely reflects other roles for this actin regulator than at N-cadherin junctions. While linkage of cortactin to N-cadherin junctions occurred in the EQ zone, cortactin was highly associated with N-cadherin complexes only in the elongating lens fiber cells of the FP region (Fig. 6A). This study shows that in the embryonic lens cortactin was recruited to N-cadherin junctions specifically at times of rapid formation of the actin cytoskeleton and elongation of lens fiber cells (the FP zone). The high level of association of cortactin with N-cadherin junctions in this region of lens fiber cell differentiation and morphogenesis places this actin regulator in position to be a key mediator of N-cadherin-directed actin cytoskeletal dynamics during lens fiber cell morphogenesis in vivo.

The results of the co-immunoprecipitation studies suggested that as lens cells begin their elongation and convergent extension in the TZ region cortactin would become highly localized along the cell–cell borders of these young, differentiating lens fiber cells. To examine this question we performed confocal analysis on isolated lens epithelial explants following immunostaining for cortactin. In undifferentiated lens epithelial cells, where biochemical analysis showed that little cortactin is associated with N-cadherin junctions, cortactin was present only at the lens cells' apical-most borders and basal surfaces (Fig. 6B), paralleling the localization of F-actin in these cells (see Fig. 1E). The staining intensity of cortactin at apical cell–cell borders greatly intensified in the EQ zone (Fig. 6C), a region of the lens where cortactin also was localized to discrete sites along the cells' lateral interfaces. However, the staining intensity for cortactin along these cell–cell borders greatly diminished in an apical to basal direction. In the TZ region cortactin was highly associated all along the cells' lateral cell interfaces (Fig. 6D), paralleling the high level of association of cortactin with N-cadherin complexes that was observed in the FP zone (Fig. 6A). The strong staining pattern for cortactin along cell–cell borders of elongating lens fiber cells was consistent with a role for cortactin in the organization of actin structures that drive lens fiber cell morphogenesis.

Cortactin associates with N-cadherin junctions through p120 catenin

p120 catenin has been assigned many roles in the cell, including one of stabilizing cell–cell junctions. Cortactin can directly interact with p120 through its N-terminus, co-localizes with both p120 and actin at cell–cell junctions, and depletion of p120 causes the loss of cortactin and its binding partner Arp3 from cell–cell borders (Boguslavsky et al., 2007). Although p120 was highly expressed in the EC zone (Fig. 7A), co-immunoprecipitation analysis showed little association between this catenin and N-cadherin in these undifferentiated lens epithelial cells (Fig. 7B). p120 catenin first became highly linked to N-cadherin junctions in the EQ zone, and a high level of association persisted throughout the period of lens fiber cell morphogenesis (FP). These findings are consistent with a role for p120 in stabilizing N-cadherin junctions and as a linker between N-cadherin and cortactin. The differentiation-state specific association between p120 and cortactin was confirmed directly by immunoprecipitating p120 from extracts of isolated differentiation-specific regions of the lens and immunoblotting for cortactin (Fig. 7C). The results of this study showed that cortactin was associated with complexes containing p120 almost exclusively in the EQ and FP zones of the developing lens, a pattern that paralleled the temporal association of both p120 and cortactin with N-cadherin.

Arp3 is specifically recruited to N-cadherin junctional complexes as lens fiber cells elongate

In vitro studies have shown that cortactin can regulate cadherin-directed assembly of actin through its activation of the Arp2/3 complex (Higgs and Pollard, 2001; Uruno et al., 2001; Weaver et al., 2001). As a nucleator of actin filament assembly the Arp2/3 complex then plays a central role in the regulation of actin dynamics in a cell (Amann and Pollard, 2001; Higgs and Pollard, 2001; Machesky and Gould, 1999). Given this function, it was not surprising to find that Arp3 was expressed in all four differentiation-state specific regions of the embryonic lens; EC, EQ, FP, and FC (Fig. 8A). Co-immunoprecipitation analysis revealed; however, that the recruitment of Arp3 to N-cadherin junctions was specific to the regions of fiber cell elongation and morphogenesis (Fig. 8A). No association was detected between this actin nucleator and N-cadherin in lens epithelial cells. Arp3 was recruited specifically and highly to N-cadherin junctional complexes in the FP zone (Fig. 8A), where cell–cell adhesion zones are rapidly extended for the process of fiber cell elongation. The association

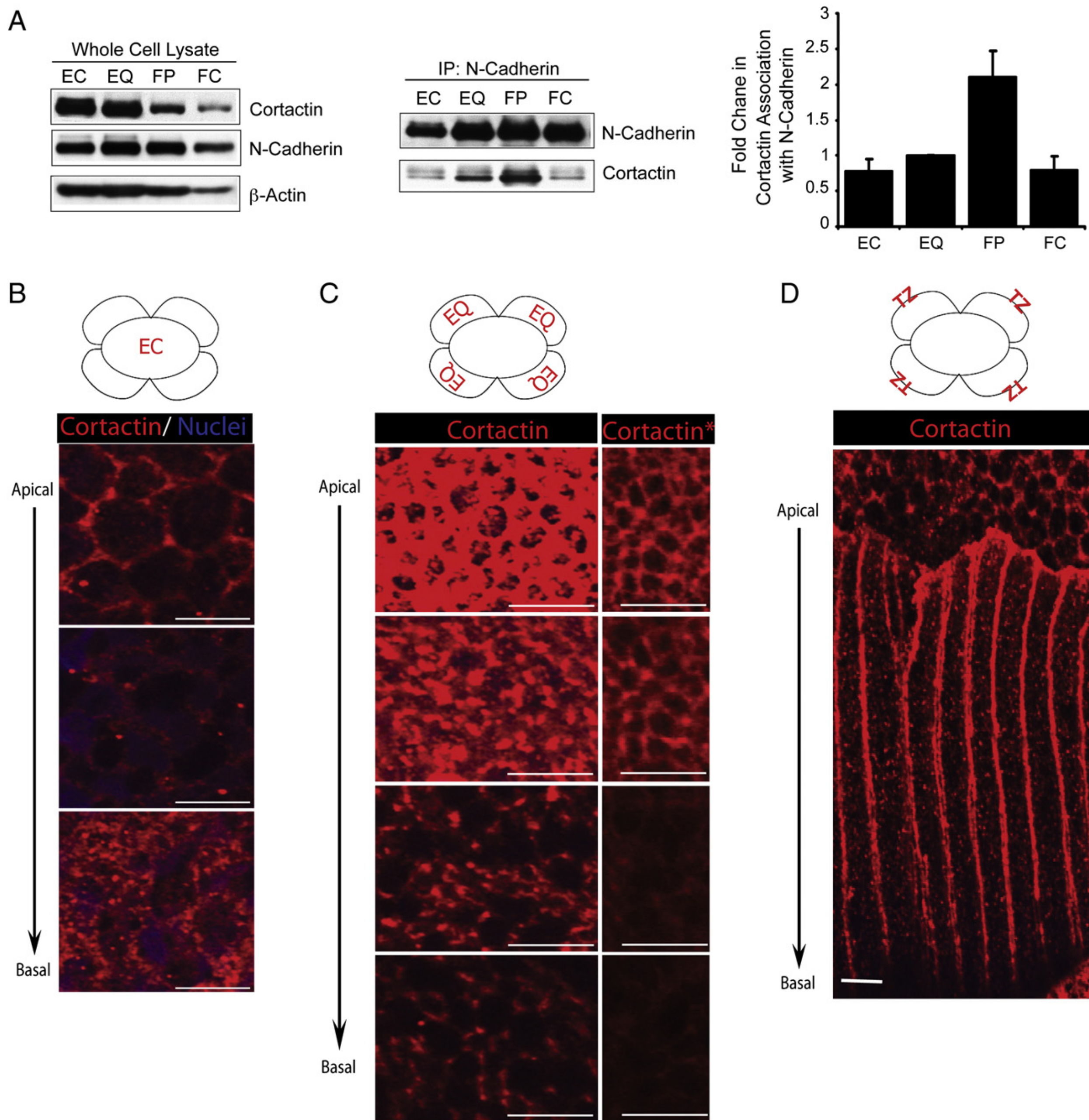

Fig. 6. Recruitment of cortactin to N-cadherin complexes supports a role for the cadherin junctions in directing actin polymerization during fiber cell differentiation. (A) Western Blot analysis of whole cell lysates showed expression of cortactin relative to N-cadherin and β-actin in each differentiation-specific zone of the E10 lens. Recruitment of cortactin to N-cadherin junctions and the differentiation-state specificity of this interaction were determined by immunoprecipitation for N-cadherin followed by Western Blot for cortactin. Densitometric analyses were plotted as the ratio of cortactin to N-cadherin, relative to EQ. Cortactin association with N-cadherin was greatest during fiber cell elongation (FP). (B–D) Confocal imaging of immunostained epithelial explants. In undifferentiated epithelial cells (B) there was cortactin associated with the cells' apical cortex, but little membrane localization along lateral cell borders. More significant staining for cortactin was observed at the cells' basal surfaces. In the EQ zone (C) cortactin became highly localized along the cells' apicolateral borders, and then extended all along the cell–cell borders of the elongating cells in the TZ region (D). All images were acquired with the same settings, with the exception of the column of panels labeled cortactin*, in which settings were optimized for imaging cortactin at the apicolateral cell–cell interfaces. Results are representative of at least 3 independent studies. Bar, 10 μm.

between Arp3 and N-cadherin remained high in the maturing fiber cells of the FC zone. We believe these are the first studies to link the recruitment of an actin nucleator to N-cadherin junctional complexes with differentiation-state specific morphogenesis in vivo.

We next investigated whether both N-cadherin/β-catenin and N-cadherin/γ-catenin junctions could serve as platforms for recruitment of cortactin and Arp3 using our double immunoprecipitation protocol (Fig. 8B). Because Arp3 is exclusively linked to N-cadherin junctions in differentiating lens fiber cells, for these studies lenses were micro-dissected only into epithelial (E) and fiber (F) cell fractions. The results showed that in lens epithelial cells cortactin linked exclusively to N-cadherin/β-catenin junctions, while in lens fiber regions cortactin linked to both N-cadherin/β-catenin and N-cadherin/γ-catenin junctions. Arp3 was recruited to N-cadherin junctions of both the β- and γ-catenin types, but only in differentiating fiber cells. As Arp3 antibodies did not work well for immunostaining in the chick embryo lens, we examined the localization of Arp3 in young mouse lenses, where the process of adding new, elongating fiber cells takes place in the cortical fiber region. In cross-sections of these hexagonally-shaped lens fiber cells Arp3 co-localized with N-cadherin and F-actin at the cell vertices, while at the lateral cell borders of these cells N-cadherin co-localized with F-actin but not Arp3 (Fig. 8C). Indeed,

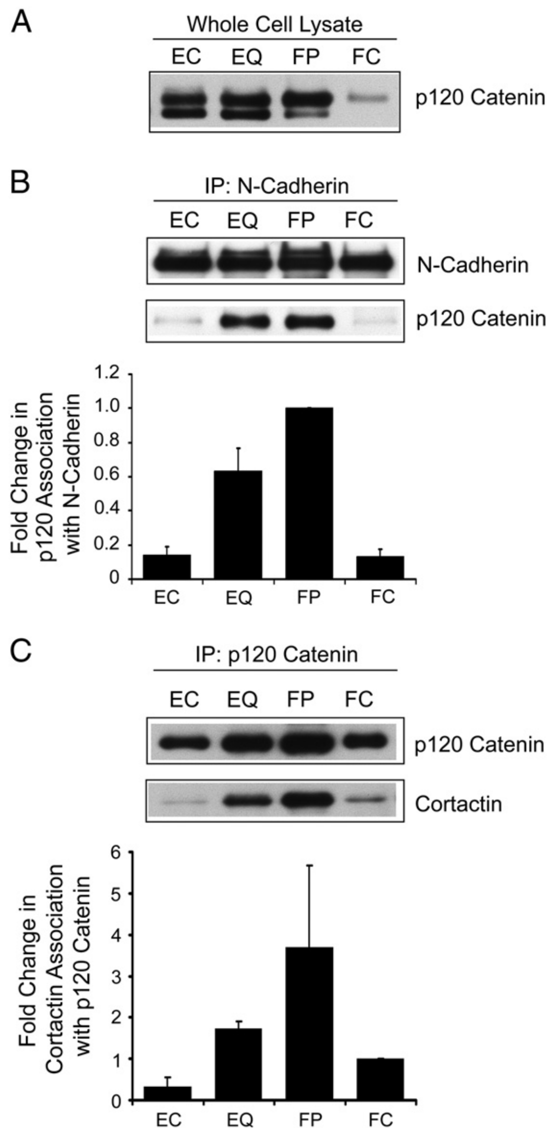

Fig. 7. Cortactin association with p120 catenin is a likely mechanism for cortactin association with N-cadherin complexes. (A) Western Blot analysis of whole cell lysates demonstrated the expression of p120 catenin in each region of lens cell differentiation. (B) Differentiation-state specific association of p120 with N-cadherin was examined by immunoprecipitation for N-cadherin followed by Western Blot for p120 catenin. Densitometric analyses showed the ratio of p120 to N-cadherin, relative to FP. p120 catenin was highly recruited to N-cadherin junctions in the EQ and FP zones of the lens. (C) Immunoprecipitation for p120 catenin followed by Western Blot for cortactin, analyzed by densitometric analysis and plotted as the ratio of cortactin to p120 relative to FC. The results showed that, in a similar pattern to the differentiation-state specific association of p120 with N-cadherin, cortactin is highly associated with p120 catenin in differentiating lens cells. Results are representative of at least 3 independent studies.

the assembly of actin filaments at lens cell–cell adhesion sites is likely critical for the extension of cadherin adhesion zones that generate the forces necessary for fiber cell morphogenesis.

N-cadherin function-blocking antibody inhibits association of α-catenin with N-cadherin junctions, organization of a cortical actin cytoskeleton and elongation of fiber cells

The recruitment of cortactin and Arp3 to N-cadherin junctions as lens epithelial cells elongate to form differentiated lens fiber cells provided evidence that there was a link, in vivo, between N-cadherin junction formation, actin assembly and cell elongation. Therefore, we designed studies to provide functional evidence of the role of N-cadherin junction maturation in directing formation of a cortical actin cytoskeleton that drives fiber cell elongation. For these studies ex vivo lens explants that contain cells in the EC, EQ and transition zones were prepared as described in Fig. 1C. These ex vivo cultures were particularly well-suited to this study because they contain cells undergoing N-cadherin junctional maturation (the EQ zone) and cells that have begun their elongation/morphogenesis into lens fiber cells (the TZ region). The explants were cultured in the presence of the N-cadherin function-blocking antibody NCD-2, which has been shown to effectively inhibit N-cadherin junction formation in primary cultures of lens epithelial cells (Ferreira-Cornwell et al., 2000). As NCD-2 is a rat monoclonal antibody, control cultures were exposed to rat IgG.

Co-immunoprecipitation analysis (immunoprecipitate: N-cadherin, Blot: α-catenin) performed after a four hour incubation of the explants in the presence of NCD-2 revealed that inhibition/disruption of N-cadherin junctions blocked the linkage of α-catenin with N-cadherin receptor complexes, with no effect on the association of β-catenin with these cadherin complexes (Fig. 9A). Confocal imaging of the NCD-2-treated explants following immunostaining for α-catenin confirmed that NCD-2 had localized to cell–cell junctions during the incubation period and showed that α-catenin was lost from cell–cell junctions in response to blocking N-cadherin function (Fig. 9B). The localization of NCD-2 (and rat IgG) in Fig. 9 was determined by the binding of fluorescence-conjugated secondary antibody alone, no additional primary antibody to N-cadherin was used for this analysis. In control cultures there was no binding of IgG either to lens epithelial cells (Fig. 9B) or to cells in the transition zone (Fig. 9C). Long-term destabilization of N-cadherin junctions following a twenty-four hour exposure to NCD-2 not only interfered with β-catenin recruitment to N-cadherin junctions but also resulted in significant downregulation of N-cadherin expression (Fig. 9A).

Strikingly, association of N-cadherin function-blocking antibody with N-cadherin junctions at lateral cell–cell interfaces of the elongating fiber cells in the transition zone caused disruption of the actin cytoskeleton at these sites (Fig. 9C). Importantly, blocking N-cadherin function at cell–cell junctions in this zone where lens cell morphogenesis begins also interfered with elongation of these newly differentiating lens fiber cells (Fig. 9D). These results provide the first functional link between α-catenin and the stabilization of N-cadherin junctions and between N-cadherin junction formation, assembly of a cortical actin cytoskeleton and cell elongation during tissue morphogenesis in vivo.

Discussion

The formation of mature cadherin junctions and the assembly of the actin cytoskeleton are both linked to processes essential to cell differentiation and tissue morphogenesis (Gumbiner, 1996, 2005; Hatta and Takeichi, 1986; Jamora and Fuchs, 2002; Larue et al., 1996; Takeichi, 1995b; Wheelock and Jensen, 1992). For a long time the relationship between these junctions and the actin cytoskeleton has been defined in terms of a direct role for the cytoskeleton in determining cadherin junction maturation. Now a number of in vitro studies have challenged this long-standing belief, demonstrating that cadherin junctions can mature independently of the actin cytoskeleton and that the maturation of cadherin junctions itself may be a principal element in driving actin filament formation and the organization of the membrane-associated cytoskeleton associated with cell movement and morphogenesis (Bajpai et al., 2008, 2009; Ehrlich et al., 2002; Helwani et al., 2004; Kovacs et al., 2002b; Vasioukhin et al., 2000). Those discoveries most important to shifting the paradigm of the relationship between cadherin junctions, the actin cytoskeleton and cell behavior include the following: 1) α-catenin cannot bind simultaneously to β-catenin and the actin cytoskeleton, a finding that challenges the concept that α-catenin directly links cadherins to the actin cytoskeleton to stabilize cadherin junctions (Drees et al., 2005; Yamada et al., 2005); 2) α-catenin association with E-cadherin complexes is sufficient for stabilization of

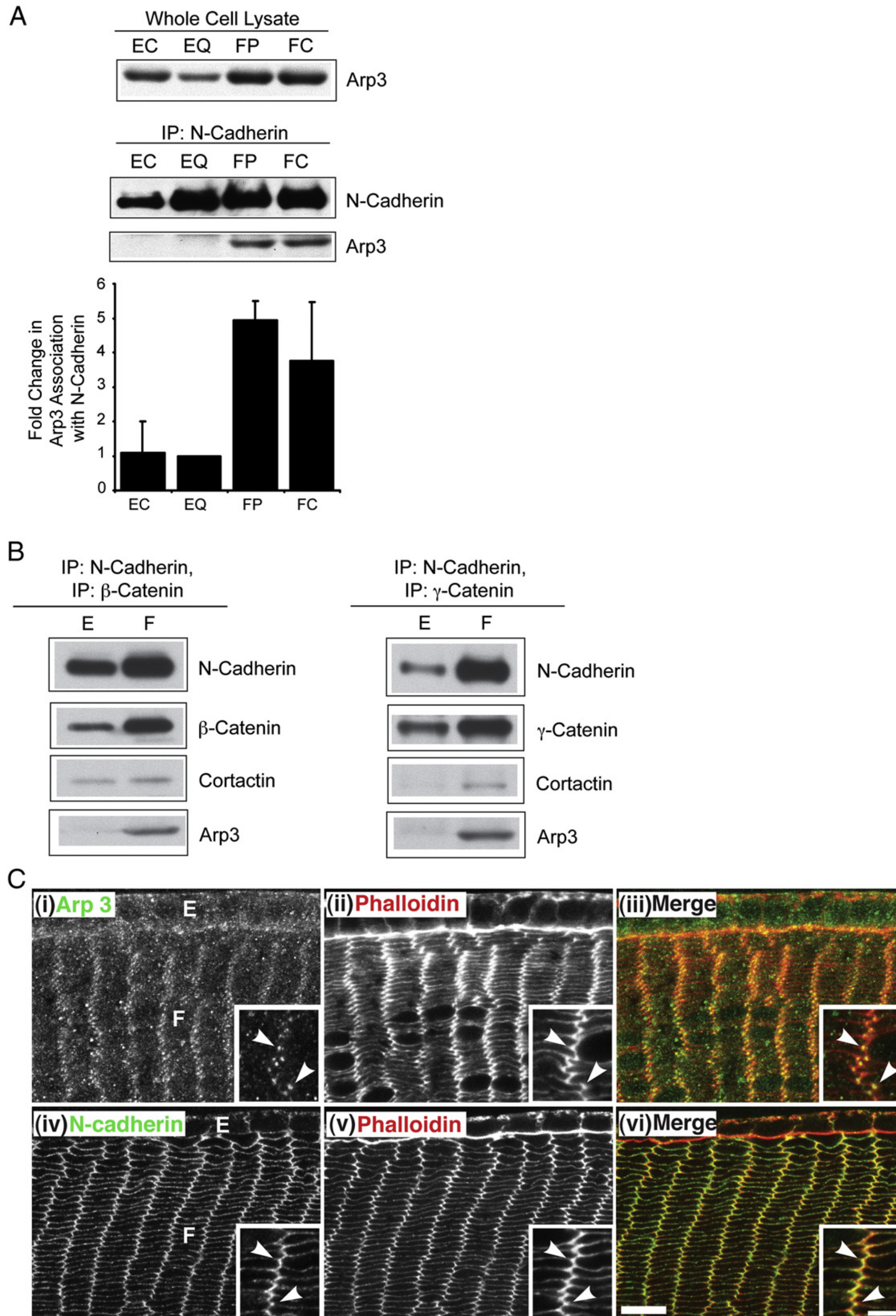

Fig. 8. Arp3, a regulator of actin filament assembly, is recruited to mature N-cadherin junctions in regions of fiber cell elongation. (A) Western Blot analysis of whole cell lysates revealed that Arp3 was most highly expressed in differentiating lens fiber cells. Immunoprecipitation of N-cadherin followed by Western Blot analysis for Arp3 showed this actin nucleator was associated with N-cadherin junctions only in fiber cells. Densitometric analysis of the ratio of Arp3 to N-cadherin, relative to EQ, revealed a nearly 5-fold increase in the association of Arp3 with N-cadherin in lens fiber cells. (B) N-cadherin/β-catenin and N-cadherin/γ-catenin complexes were isolated from lens epithelial (E) and fiber cells (F) using the double immunoprecipitation approach and the association of cortactin and Arp3 with these complexes was determined by Western Blot analysis. Cortactin and Arp3 associated with both N-cadherin/β-catenin and N-cadherin/γ-catenin junctions, Arp3 exclusively in lens fiber cells. (C) Equatorial sections of 1 month-old mouse lenses immunostained for Arp3 or N-cadherin, and double-stained with phalloidin to visualize F-actin showed that Arp3 and N-cadherin co-localized with bright F-actin at vertices of fiber cell hexagonal profiles. N-cadherin, but not Arp3, also was associated with F-actin along the entire fiber cell membrane. Merges: Arp3 or N-cadherin (green), F-actin (red). The lens epithelium (E) is located at the top of the images, just above the lens fiber cells (F). Arrowheads in insets, vertices. Bar, 11 µm; (insets) 4 µm. Results are representative of at least 3 independent studies.

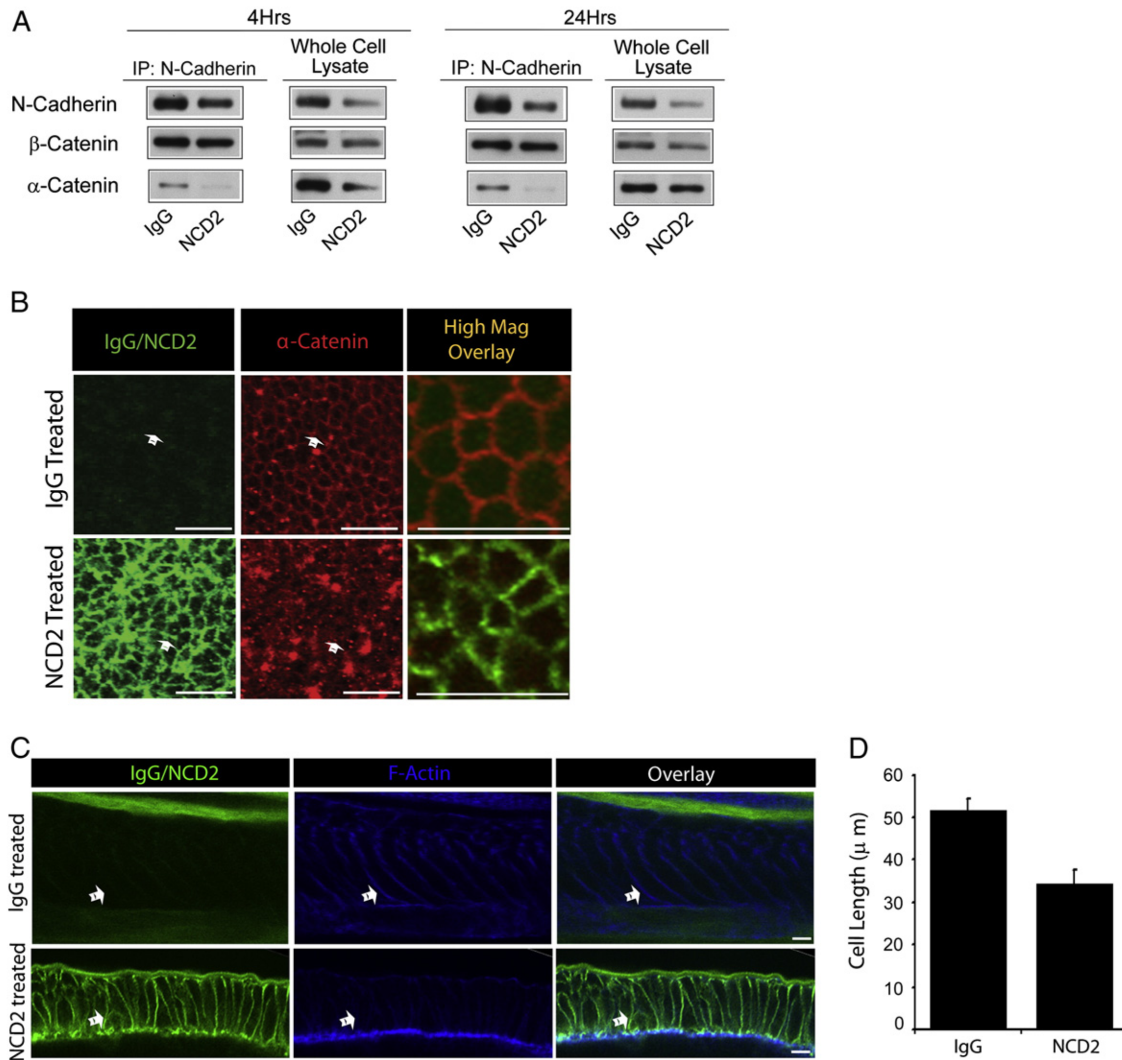

Fig. 9. Function-blocking antibody to N-cadherin blocks α-catenin from association with junctional complexes, assembly of a cortical actin cytoskeleton, and fiber cell elongation. Epithelial explants were cultured in the presence of the N-cadherin function-blocking antibody NCD-2 or control rat IgG. (A) The effect of N-cadherin function-blocking antibody on the association of α-catenin and β-catenin with N-cadherin complexes was determined by co-immunoprecipitation analysis (immunoprecipitate: N-cadherin, Blot: α-catenin or β-catenin). Results were compared to control explants exposed to rat IgG. Four-hour exposure to NCD-2 resulted in reduced association of α-catenin with N-cadherin complexes with no effect on the link between N-cadherin and β-catenin (left panels). Long-term exposure to NCD-2 (twenty-four hours, right panels) blocked association of α-catenin with N-cadherin junctions and, in addition, resulted in downregulation of N-cadherin expression. (B) Immunolocalization of α-catenin in epithelial explants exposed to NCD-2 or control IgG for 4 h. Following fixation the localization of the NCD-2 antibody or IgG was determined by incubation with a fluorescent-conjugated secondary antibody and the samples were double-stained with antibody to α-catenin. Right hand panels are high magnification overlays, the top panel a co-localization of control IgG antibody with α-catenin, the bottom panel a co-localization of NCD-2 antibody with α-catenin. Samples were viewed by confocal microscopy and Z-stacks collected. Images presented represent a single optical plane within the Z-stack in the apicolateral region of the cells. The results showed a loss of α-catenin from cell–cell interfaces of explants treated with NCD-2. Results are representative of at least 3 independent studies; bar, 10 μm. (C) Cells in the transition zone (TZ) of NCD-2 treated explants (after a four exposure) were stained with fluorescent-conjugated phalloidin to visualize actin (blue), and fluorescent-conjugated secondary antibodies to localize NCD-2 or control IgG (green). Imaging of these ex vivo explants by confocal microscopy showed that NCD-2 had localized to N-cadherin junctions at cell–cell interfaces of differentiating fiber cells in the TZ, and disrupted the formation of the cortical actin skeleton in these differentiating fiber cells. Images were collected as Z-stacks and data shown represent a single optical plane within the Z-stack; bar, 10 μm. (D) Measurements of fiber cell length in the TZ region of explants exposed to NCD-2 or control rat IgG for 4 h showed that destabilization of N-cadherin junctions with NCD-2 blocked the elongation of lens fiber cells, demonstrating that N-cadherin-linked actin organization was responsible for lens fiber cell morphogenesis. All results are representative of at least 3 independent studies. Arrows denote the same position in double-stained images.

these junctions through a mechanism independent of the actin cytoskeleton (Bajpai et al., 2008, 2009) and 3) the formation of E-cadherin junctions induces recruitment of the actin regulators cortactin and Arp2/3 directly to these junctions to signal assembly of actin filaments (Ehrlich et al., 2002; Helwani et al., 2004; Kovacs et al., 2002b; Vasioukhin et al., 2000). In studies with a primary lens cell culture system we had shown previously that the maturation of N-cadherin junctions is required for the differentiation of lens epithelial cells (Ferreira-Cornwell et al., 2000; Wheelock and Jensen, 1992). Now, in studies focused on the embryonic lens, we provide in vivo evidence of a direct relationship between N-cadherin junctional maturation, actin cytoskeletal assembly and cell morphogenesis.

The spectrum of differentiation-specific morphogenesis that exists in the embryonic lens provided a unique window into the complexity of cadherin junction formation and function in vivo (modeled in Fig. 10). Confocal microscopy imaging, an important tool in this analysis, revealed that nascent cadherin junctions, a junctional organization previously described in cell culture (Ferreira-Cornwell et al., 2000; Vasioukhin et al., 2000; Wheelock and Jensen, 1992), were indeed a feature of undifferentiated epithelia in vivo. These nascent N-cadherin junctions appeared as puncta formed at the contact sites of lamellipodial-like protrusions extended between opposing lateral cell membranes of undifferentiated lens epithelial cells. These nascent junctions matured and zipped up the lateral cell

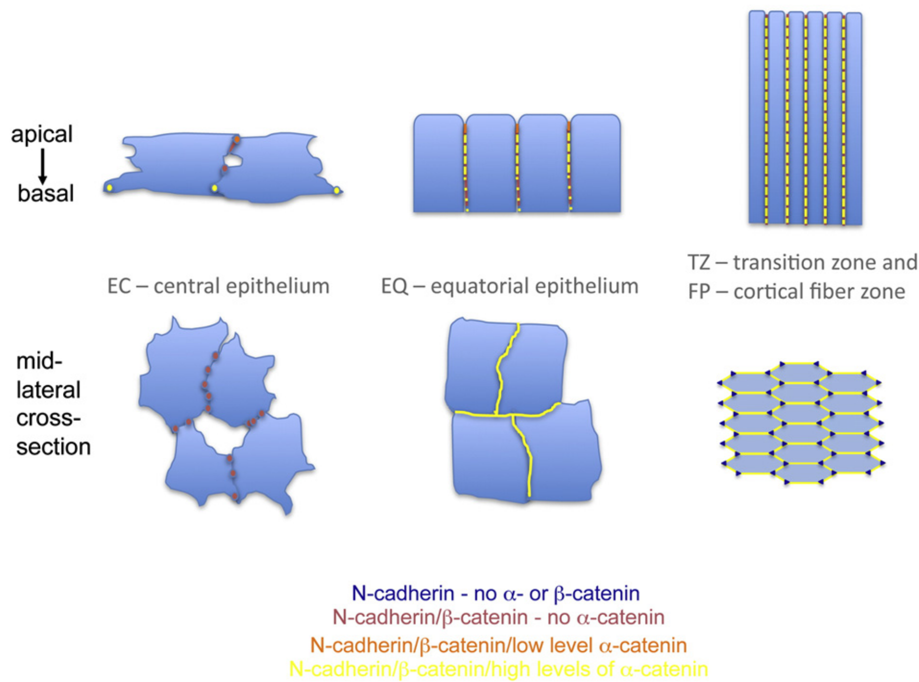

Fig. 10. α-catenin in the maturation of N-cadherin/β-catenin junctions with lens epithelial cell differentiation. Lens epithelial cells are diagrammed through their elongation into lens fiber cells in the transition zone, focusing on the role of α-catenin in the maturation of N-cadherin junctions along lateral cell borders. Each color represents a distinct N-cadherin junctional complex: blue — N-cadherin junctions with no associated α- or β-catenin; red — N-cadherin/β-catenin junctions with no associated α-catenin; orange — N-cadherin/β-catenin junctions with a low level of associated α-catenin; yellow — N-cadherin/β-catenin junctions with a high level of associated α-catenin.

interfaces of lens epithelial cells as they initiated their differentiation, a process that preceded the elongation that characterizes lens fiber cell morphogenesis. The maturation of N-cadherin junctions in lens epithelial cells involved their association with α-catenin (Fig. 10), paralleling the mechanism recently described for E-cadherin junctional maturation in vitro (Bajpai et al., 2008, 2009). The association of α-catenin with these junctional complexes was facilitated by the dephosphorylation of β-catenin, which appeared to occur through the action of SHP-2, a tyrosine phosphatase that can bind directly to cadherin-linked β-catenin (Ukropec et al., 2000). Exposure to an N-cadherin function-blocking antibody previously shown to interfere with junctional stabilization (Ferreira-Cornwell et al., 2000) blocked this association of α-catenin with N-cadherin complexes, providing a functional connection between α-catenin recruitment to N-cadherin junctions and maturation of N-cadherin junctions in the embryonic lens.

While culture studies have shown that cadherin junctions can function as sites of actin filament assembly (Ehrlich et al., 2002; Helwani et al., 2004; Kovacs et al., 2002b; Vasioukhin et al., 2000), particularly compelling questions have remained regarding the mechanisms that tie together cadherin junction formation, actin cytoskeletal assembly and the processes of cell and tissue morphogenesis in vivo. Previous studies have implicated both N-cadherin junctions and actin dynamics as key regulators of fiber cell morphogenesis in the lens. Defects in the elongation of lens fiber cells have been reported in mice with lens-specific deletions of either N-cadherin (Pontoriero et al., 2009) or β-catenin (Cain et al., 2008), and in both of these knockouts the lens cells also exhibit alterations in the actin cytoskeleton. In transgenic mice expressing RhoGDI, an inhibitor of the RhoGTPases that mediate actin filament assembly, lens fiber cells fail to elongate properly (Maddala et al., 2008), while in lens cell primary culture, blocking actin stress fiber assembly while promoting formation of a cortical actin cytoskeleton is sufficient to induce lens cell differentiation (Weber and Menko, 2006). In this report, we show that N-cadherin junctions are de facto sites of actin filament assembly in vivo and that the mechanism for organization of the cortical actin structures that drive elongation/morphogenesis of lens fiber cells in vivo involves recruitment of the actin-nucleating factor Arp3 to N-cadherin/cortactin complexes. Arp3 links directly to cortactin (Higgs and Pollard, 2001; Uruno et al., 2001; Weaver et al., 2001; Weed et al., 2000), an actin polymerization factor whose own association with N-cadherin complexes in the embryonic lens was temporally coincident with the assembly of mature N-cadherin junctions. This finding suggests that the cadherin junctional maturation process itself signals the recruitment of cortactin. The association of cortactin with N-cadherin junctions was mediated by p120 catenin, a molecular regulator with a role in stabilizing cell–cell junctions (Iyer et al., 2004) that binds both to the juxtamembrane domain of classical cadherins (Kowalczyk and Reynolds, 2004) and to the N-terminus of cortactin (Boguslavsky et al., 2007).

The assembly of actin filaments by Arp2/3 has been implicated in generating the forces that promote cell surface protrusion (Pollard et al., 2000; Ren et al., 2009), a process ideal for creating the forces at cell–cell interfaces that drive the dramatic elongation of lens fiber cells that is essential to lens morphogenetic differentiation. In the embryonic lens Arp3 was recruited to N-cadherin junctions specifically when lens cells begin this morphogenetic differentiation. Studies with an N-cadherin function-blocking antibody showed for the first time that assembly of mature N-cadherin junctions is required for assembly of the cortical actin cytoskeleton of lens fiber cells and for fiber cell elongation, the central feature of lens morphogenesis. Our findings support a mechanism in which N-cadherin-directed actin filament assembly generates the forces that extend cell–cell adhesion zones during fiber cell elongation. Cadherin-directed assembly of actin filaments can generate cytoskeletal forces that are transmitted to cell shape changes as a result of anchoring cytoskeletal networks to sites of cell–cell adhesions (Chen et al., 1997; Chicurel et al., 1998; Liu and Chen, 2007; McBeath et al., 2004). As such, the coordinated cadherin-directed assembly and organization of actin can provide the driving forces necessary for cell elongation and morphogenesis in the developing lens. We believe this to be a fundamental property of classical cadherins, and that cadherin/cortactin/Arp3-directed

assembly of actin filaments provides the mechanism by which cadherins regulate tissue morphogenesis in vivo.

Acknowledgments

This work was supported by NIH grants EY10577, EY014258 and EY014798 to ASM, T32E5007283 to ML and HL083464 to VMF. We thank Iris Wolff for technical assistance, Janice L. Walker for intellectual support and Terry Hyslop for statistical advice.

References

Amann, K.J., Pollard, T.D., 2001. The Arp2/3 complex nucleates actin filament branches from the sides of pre-existing filaments. Nat. Cell Biol. 3, 306–310.
Bajpai, S., Correia, J., Feng, Y., Figueiredo, J., Sun, S.X., Longmore, G.D., Suriano, G., Wirtz, D., 2008. {alpha}-Catenin mediates initial E-cadherin-dependent cell–cell recognition and subsequent bond strengthening. Proc. Natl Acad. Sci. USA 105, 18331–18336.
Bajpai, S., Feng, Y., Krishnamurthy, R., Longmore, G.D., Wirtz, D., 2009. Loss of alpha-catenin decreases the strength of single E-cadherin bonds between human cancer cells. J. Biol. Chem. 284, 18252–18259.
Balsamo, J., Leung, T., Ernst, H., Zanin, M.K., Hoffman, S., Lilien, J., 1996. Regulated binding of PTP1B-like phosphatase to N-cadherin: control of cadherin-mediated adhesion by dephosphorylation of beta-catenin. J. Cell Biol. 134, 801–813.
Boguslavsky, S., Grosheva, I., Landau, E., Shtutman, M., Cohen, M., Arnold, K., Feinstein, E., Geiger, B., Bershadsky, A., 2007. p120 catenin regulates lamellipodial dynamics and cell adhesion in cooperation with cortactin. Proc. Natl Acad. Sci. USA 104, 10882–10887.
Cain, S., Martinez, G., Kokkinos, M.I., Turner, K., Richardson, R.J., Abud, H.E., Huelsken, J., Robinson, M.L., de Iongh, R.U., 2008. Differential requirement for beta-catenin in epithelial and fiber cells during lens development. Dev. Biol. 321, 420–433.
Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.M., Ingber, D.E., 1997. Geometric control of cell life and death. Science 276, 1425–1428.
Chicurel, M.E., Chen, C.S., Ingber, D.E., 1998. Cellular control lies in the balance of forces. Curr. Opin. Cell Biol. 10, 232–239.
Daniel, J.M., Reynolds, A.B., 1997. Tyrosine phosphorylation and cadherin/catenin function. Bioessays 19, 883–891.
Drees, F., Pokutta, S., Yamada, S., Nelson, W.J., Weis, W.I., 2005. Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. Cell 123, 903–915.
Ehrlich, J.S., Hansen, M.D., Nelson, W.J., 2002. Spatio-temporal regulation of Rac1 localization and lamellipodia dynamics during epithelial cell–cell adhesion. Dev. Cell 3, 259–270.
Ferreira-Cornwell, M.C., Veneziale, R.W., Grunwald, G.B., Menko, A.S., 2000. N-cadherin function is required for differentiation-dependent cytoskeletal reorganization in lens cells in vitro. Exp. Cell Res. 256, 237–247.
Gumbiner, B.M., 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 84, 345–357.
Gumbiner, B.M., 2005. Regulation of cadherin-mediated adhesion in morphogenesis. Nat. Rev. Mol. Cell Biol. 6, 622–634.
Hatta, K., Takeichi, M., 1986. Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. Nature 320, 447–449.
Helwani, F.M., Kovacs, E.M., Paterson, A.D., Verma, S., Ali, R.G., Fanning, A.S., Weed, S.A., Yap, A.S., 2004. Cortactin is necessary for E-cadherin-mediated contact formation and actin reorganization. J. Cell Biol. 164, 899–910.
Higgs, H.N., Pollard, T.D., 2001. Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. Annu. Rev. Biochem. 70, 649–676.
Hinck, L., Nathke, I.S., Papkoff, J., Nelson, W.J., 1994. Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. J. Cell Biol. 125, 1327–1340.
Huber, O., Krohn, M., Kemler, R., 1997. A specific domain in alpha-catenin mediates binding to beta-catenin or plakoglobin. J. Cell Sci. 110 (Pt 15), 1759–1765.
Iyer, S., Ferreri, D.M., DeCocco, N.C., Minnear, F.L., Vincent, P.A., 2004. VE-cadherin–p120 interaction is required for maintenance of endothelial barrier function. Am. J. Physiol. Lung Cell. Mol. Physiol. 286, L1143–L1153.
Jamora, C., Fuchs, E., 2002. Intercellular adhesion, signalling and the cytoskeleton. Nat. Cell Biol. 4, E101–E108.
Kemler, R., 1993. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. Trends Genet. 9, 317–321.
Kobielak, A., Fuchs, E., 2004. Alpha-catenin: at the junction of intercellular adhesion and actin dynamics. Nat. Rev. Mol. Cell Biol. 5, 614–625.
Kovacs, E.M., Ali, R.G., McCormack, A.J., Yap, A.S., 2002a. E-cadherin homophilic ligation directly signals through Rac and phosphatidylinositol 3-kinase to regulate adhesive contacts. J. Biol. Chem. 277, 6708–6718.
Kovacs, E.M., Goodwin, M., Ali, R.G., Paterson, A.D., Yap, A.S., 2002b. Cadherin-directed actin assembly: E-cadherin physically associates with the Arp2/3 complex to direct actin assembly in nascent adhesive contacts. Curr. Biol. 12, 379–382.
Kovacs, E.M., Yap, A.S., 2002. The web and the rock: cell adhesion and the ARP2/3 complex. Dev. Cell 3, 760–761.
Kowalczyk, A.P., Reynolds, A.B., 2004. Protecting your tail: regulation of cadherin degradation by p120-catenin. Curr. Opin. Cell Biol. 16, 522–527.
Larue, L., Antos, C., Butz, S., Huber, O., Delmas, V., Dominis, M., Kemler, R., 1996. A role for cadherins in tissue formation. Development 122, 3185–3194.
Leonard, M., Chan, Y., Menko, A.S., 2008. Identification of a novel intermediate filament-linked N-cadherin/gamma-catenin complex involved in the establishment of the cytoarchitecture of differentiated lens fiber cells. Dev. Biol. 319, 298–308.
Lilien, J., Balsamo, J., Arregui, C., Xu, G., 2002. Turn-off, drop-out: functional state switching of cadherins. Dev. Dyn. 224, 18–29.
Liu, W.F., Chen, C.S., 2007. Cellular and multicellular form and function. Adv. Drug Deliv. Rev. 59, 1319–1328.
Machesky, L.M., Gould, K.L., 1999. The Arp2/3 complex: a multifunctional actin organizer. Curr. Opin. Cell Biol. 11, 117–121.
Maddala, R., Reneker, L.W., Pendurthi, B., Rao, P.V., 2008. Rho GDP dissociation inhibitor-mediated disruption of Rho GTPase activity impairs lens fiber cell migration, elongation and survival. Dev. Biol. 315, 217–231.
McBeath, R., Pirone, D.M., Nelson, C.M., Bhadriraju, K., Chen, C.S., 2004. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev. Cell 6, 483–495.
Nowak, R.B., Fischer, R.S., Zoltoski, R.K., Kuszak, J.R., Fowler, V.M., 2009. Tropomodulin1 is required for membrane skeleton organization and hexagonal geometry of fiber cells in the mouse lens. J. Cell Biol. 186, 915–928.
Ozawa, M., Kemler, R., 1998. Altered cell adhesion activity by pervanadate due to the dissociation of alpha-catenin from the E-cadherin.catenin complex. J. Biol. Chem. 273, 6166–6170.
Perez-Moreno, M., Jamora, C., Fuchs, E., 2003. Sticky business: orchestrating cellular signals at adherens junctions. Cell 112, 535–548.
Pollard, T.D., Blanchoin, L., Mullins, R.D., 2000. Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. Annu. Rev. Biophys. Biomol. Struct. 29, 545–576.
Pontoriero, G.F., Smith, A.N., Miller, L.A., Radice, G.L., West-Mays, J.A., Lang, R.A., 2009. Co-operative roles for E-cadherin and N-cadherin during lens vesicle separation and lens epithelial cell survival. Dev. Biol. 326, 403–417.
Ren, G., Crampton, M.S., Yap, A.S., 2009. Cortactin: coordinating adhesion and the actin cytoskeleton at cellular protrusions. Cell Motil. Cytoskeleton 66, 865–873.
Roura, S., Miravet, S., Piedra, J., Garcia de Herreros, A., Dunach, M., 1999. Regulation of E-cadherin/catenin association by tyrosine phosphorylation. J. Biol. Chem. 274, 36734–36740.
Takeichi, M., 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. [Review]. Science 251, 1451–1455.
Takeichi, M., 1995a. Morphogenetic roles of classic cadherins. Curr. Opin. Cell Biol. 7, 619–627.
Takeichi, M., 1995b. Morphogenetic roles of classical cadherins. Curr. Opin. Cell Biol. 5, 806–811.
Ukropec, J.A., Hollinger, M.K., Salva, S.M., Woolkalis, M.J., 2000. SHP2 association with VE-cadherin complexes in human endothelial cells is regulated by thrombin. J. Biol. Chem. 275, 5983–5986.
Uruno, T., Liu, J., Zhang, P., Fan, Y., Egile, C., Li, R., Mueller, S.C., Zhan, X., 2001. Activation of Arp2/3 complex-mediated actin polymerization by cortactin. Nat. Cell Biol. 3, 259–266.
Vasioukhin, V., Bauer, C., Yin, M., Fuchs, E., 2000. Directed actin polymerization is the driving force for epithelial cell–cell adhesion. Cell 100, 209–219.
Verma, S., Shewan, A.M., Scott, J.A., Helwani, F.M., den Elzen, N.R., Miki, H., Takenawa, T., Yap, A.S., 2004. Arp2/3 activity is necessary for efficient formation of E-cadherin adhesive contacts. J. Biol. Chem. 279, 34062–34070.
Wadham, C., Gamble, J.R., Vadas, M.A., Khew-Goodall, Y., 2003. The protein tyrosine phosphatase Pez is a major phosphatase of adherens junctions and dephosphorylates beta-catenin. Mol. Biol. Cell 14, 2520–2529.
Walker, J.L., Menko, A.S., 1999. alpha6 Integrin is regulated with lens cell differentiation by linkage to the cytoskeleton and isoform switching. Dev. Biol. 210, 497–511.
Walker, J.L., Zhang, L., Zhou, J., Woolkalis, M.J., Menko, A.S., 2002. Role for alpha 6 integrin during lens development: evidence for signaling through IGF-1R and ERK. Dev. Dyn. 223, 273–284.
Weaver, A.M., Karginov, A.V., Kinley, A.W., Weed, S.A., Li, Y., Parsons, J.T., Cooper, J.A., 2001. Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation. Curr. Biol. 11, 370–374.
Weber, G.F., Menko, A.S., 2006. Actin filament organization regulates the induction of lens cell differentiation and survival. Dev. Biol. 295, 714–729.
Weed, S.A., Karginov, A.V., Schafer, D.A., Weaver, A.M., Kinley, A.W., Cooper, J.A., Parsons, J.T., 2000. Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex. J. Cell Biol. 151, 29–40.
Wheelock, M.J., Jensen, P.J., 1992. Regulation of keratinocyte intercellular junction organization and epidermal morphogenesis by E-cadherin. J. Cell Biol. 117, 415–425.
Xu, L., Overbeek, P.A., Reneker, L.W., 2002. Systematic analysis of E-, N- and P-cadherin expression in mouse eye development. Exp. Eye Res. 74, 753–760.
Yamada, S., Pokutta, S., Drees, F., Weis, W.I., Nelson, W.J., 2005. Deconstructing the cadherin–catenin–actin complex. Cell 123, 889–901.