Cortactin Promotes Migration and Platelet-derived Growth Factor-induced Actin Reorganization by Signaling to Rho-GTPases

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Dynamic actin rearrangements are initiated and maintained by actin filament nucleators, including the Arp2/3-complex. This protein assembly is activated *in vitro* by distinct nucleation-promoting factors such as Wiskott-Aldrich syndrome protein/Scar family proteins or cortactin, but the relative *in vivo* functions of each of them remain controversial. Here, we report the conditional genetic disruption of murine cortactin, implicated previously in dynamic actin reorganizations driving lamellipodium protrusion and endocytosis. Unexpectedly, cortactin-deficient cells showed little changes in overall cell morphology and growth. Ultrastructural analyses and live-cell imaging studies revealed unimpaired lamellipodial architecture, Rac-induced protrusion, and actin network turnover, although actin assembly rates in the lamellipodium were modestly increased. In contrast, platelet-derived growth factor-induced actin reorganization and Rac activation were impaired in cortactin null cells. In addition, cortactin deficiency caused reduction of Cdc42 activity and defects in random and directed cell migration. Reduced migration of cortactin null cells could be restored, at least in part, by active Rac and Cdc42 variants. Finally, cortactin removal did not affect the efficiency of receptor-mediated endocytosis. Together, we conclude that cortactin is fully dispensable for Arp2/3-complex activation during lamellipodia protrusion or clathrin pit endocytosis. Furthermore, we propose that cortactin promotes cell migration indirectly, through contributing to activation of selected Rho-GTPases.

INTRODUCTION

Cell migration is a complex process requiring the coordinated activities of multiple cellular machines, driving actin polymerization, actin-myosin II-based force generation, and coupling to the extracellular matrix. However, the relative contribution of each of these machines to the different steps in the motility cycle is just beginning to emerge. Irrespective of the complexity of coordination of these activities, it is commonly agreed that protrusion at the cell front is initiated by localized actin polymerization, to form structures such as lamellipodia or ruffles (Small *et al.*, 2002; Pollard and Borisy, 2003).

The best characterized factors driving the nucleation of actin filaments in vertebrate cells are the Arp2/3-complex and formins (Pollard, 2007). Arp2/3-complex activity is considered essential for processes as diverse as lamellipodium protrusion, actin assembly during clathrin-mediated endocytosis, podosome formation, and different types of host-pathogen interaction (Goley and Welch, 2006; Linder, 2007).

Activators of Arp2/3-complex are termed nucleation promoting factors (NPFs) and are roughly subdivided into class I and II composed of classical members Wiskott-Aldrich syndrome protein (WASP)/WASP family Verprolin-homologous protein (WAVE) proteins and cortactin, respectively (Welch and Mullins, 2002). This distinction is derived, at least in part, from their mode of interaction with actin: the WH2 domains of class I and tandem repeat regions of class II NPFs bind to monomeric and filamentous actin (F-actin), respectively (Welch and Mullins, 2002; Goley and Welch, 2006; Ammer and Weed, 2008). WASP and WAVE proteins have been studied in significant detail, including the characterization of genetic mutations in the mouse (Stradal *et al.*, 2004; Takenawa and Suetsugu, 2007). In addition, exciting recent progress included the discovery of additional class I NPFs (Linardopoulou *et al.*, 2007; Campellone *et al.*, 2008; Zuchero *et al.*, 2009), which foster the view of great versatility of Arp2/3-complex function in cells. However, the precise functions of the mammalian type II NPF cortactin have

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remained controversial (Cosen-Binker and Kapus, 2006; Ammer and Weed, 2008).

Initially identified as a tyrosine phosphorylated protein in v-Src-infected chicken embryo fibroblasts (Kanner *et al.*, 1990), cortactin comprises an amino-terminal acidic domain, mediating Arp2/3-complex binding (Weed *et al.*, 2000), followed by the F-actin binding region containing six and a half 37 amino acid repeats, an α -helical domain, a proline-rich region, and a Src homology (SH) 3 domain at the distal carboxy terminus (Wu *et al.*, 1991; Sparks *et al.*, 1996). Cortactin mostly localizes to dynamic actin structures driven by Arp2/3-complex (Kaksonen *et al.*, 2000; Frischknecht and Way, 2001; Boyle *et al.*, 2007). The SH3 domain was shown to interact with a plethora of proteins, including dynamin (McNiven *et al.*, 2000) and neural (N)-WASP (Mizutani *et al.*, 2002), pointing at potential cortactin functions in endocytosis (Zhu *et al.*, 2005) and migration (Kowalski *et al.*, 2005).

In vitro assays have shown that cortactin promotes actin assembly by simultaneously binding to Arp2/3-complex and actin filaments. It was proposed therefore that cortactin mediates both Arp2/3-dependent nucleation and stabilization of Arp2/3-induced filament branches (Weaver *et al.*, 2001). Cortactin can also activate N-WASP (Kowalski *et al.*, 2005), and both can synergistically activate Arp2/3-complex in vitro (Weaver *et al.*, 2002). However, additional interactions observed for both cortactin and N-WASP with the family of WASP-interacting proteins (WIP) (Anton *et al.*, 2007), or for N-WASP and WIP with SH3 adaptors such as Nck complicate the picture of the precise composition and biochemical activities of potential cortactin-containing complexes in vivo (Tehrani *et al.*, 2007). Furthermore, the Arp2/3-modulating function of cortactin may not be solely restricted to N-WASP-dependent processes, because cortactin accumulates in structures such as lamellipodia, which lack N-WASP (Benesch *et al.*, 2005; Ladwein and Rottner, 2008).

The cortactin family comprises the ubiquitously expressed cortactin (also called EMS in humans) and the hematopoietic HS1, which shares structural and functional features with cortactin, although its repeat region is shorter (3.5 repeats) and, as opposed to cortactin, requires the α -helical region for efficient F-actin binding (Hao *et al.*, 2005). Cortactin and HS1 have recently been implicated in the regulation of several actin-dependent processes (Cosen-Binker and Kapus, 2006; Ammer and Weed, 2008), and are not always functionally redundant (Tehrani *et al.*, 2006). Consistently, conventional HS1 knockout mice are viable, although they display defects in T-cell proliferation and negative selection (Taniuchi *et al.*, 1995), recently concluded to derive from defective actin assembly and interleukin-2 production (Gomez *et al.*, 2006; Burkhardt *et al.*, 2008). In addition, HS1 has important functions in natural killer cells (Butler *et al.*, 2008). Both cortactin and HS1 are phosphorylated on multiple sites, but the functional subtleties of these phosphorylation events are just beginning to emerge (Martinez-Quiles *et al.*, 2004; Martin *et al.*, 2006; Boyle *et al.*, 2007; Tehrani *et al.*, 2007; Butler *et al.*, 2008; Kruchten *et al.*, 2008).

Cortactin knockdown by RNA interference supports its involvement in actin-dependent processes, including specific types of host-pathogen interaction (Selbach and Backert, 2005; Cosen-Binker and Kapus, 2006) and lamellipodium protrusion, although its precise mode of function in the latter process is controversial (Bryce *et al.*, 2005; Kempiak *et al.*, 2005; Illes *et al.*, 2006; van Rossum *et al.*, 2006; Cai *et al.*, 2008), due perhaps to differential efficacy of cortactin down-regulation (Cosen-Binker and Kapus, 2006).

To resolve this issue, and to define the precise functions of cortactin in Arp2/3-dependent actin assembly in vivo, we

conditionally targeted the murine cortactin gene, and we developed and characterized fibroblast cell lines lacking cortactin.

MATERIALS AND METHODS

Antibodies

Immunoblotting was performed according to standard protocols. Commercial primary antibodies were as follows: monoclonal anti-cortactin (clone 4F-11) (Millipore, Billerica, MA); monoclonal anti-Cdc42 (clone 44) (BD Biosciences Transduction Laboratories, Heidelberg, Germany); monoclonal anti-RhoA (Cytoskeleton, Denver, CO); monoclonal anti-Rac1 (clone 23A8) (Biomol, Hamburg, Germany); polyclonal anti-AKT, polyclonal anti-pAKTser473, monoclonal anti-p-extracellular signal-regulated kinase (ERK) 1/2 (clone E10; Figure 7), polyclonal anti-pERK1/2 (Supplemental Figure 5) (all Cell Signaling Technology, Danvers, MA); polyclonal anti-ERK1/2, monoclonal anti-actin (clone AC-15), monoclonal anti-vinculin (hVIN-1) (all Sigma Chemie, Deisenhofen, Germany); polyclonal anti-dynamin 2 (Abcam, Dresden, Germany); monoclonal anti- α -tubulin (clone 3A2) (Synaptic Systems, Göttingen, Germany); and monoclonal anti-clathrin heavy chain (clone 23) (BD Biosciences Transduction Laboratories). Polyclonal anti-ArpC3 antibody was as described previously (Pistor *et al.*, 2000), and monoclonal anti-WAVE2 was kindly provided by Giorgio Scita (IFOM, Milan, Italy). Polyclonal anti-Nap1 was as described previously (Steffen *et al.*, 2004). Monoclonal anti-HS1 antibody was a kind gift from Dr. Daisuke Kitamura (Tokyo University of Science, Tokyo Japan). Monoclonal antibodies specific for cortactin were raised using purified, full-length protein and screening with extracts from control and knockout (KO) fibroblast cell lines as well as B16-F1 mouse melanoma cells expressing either full-length, enhanced green fluorescent protein (EGFP)-tagged cortactin or the EGFP-tagged N terminus of cortactin (Supplemental Figure 1). Monoclonal anti-N-WASP antibodies were as described previously (Weiss *et al.*, 2009).

Protein Purification

Full-length glutathione transferase (GST)-tagged cortactin was expressed in *Escherichia coli* strain Rossetta (Promega, Madison, WI) and purified from bacterial extracts on glutathione-conjugated agarose (Sigma Chemie) by using standard procedures. The GST tag was cleaved by incubating the purified fusion protein with PreScission protease in phosphate-buffered saline (PBS), pH 7.3, supplemented with 1 mM dithiothreitol (DTT) and 1 mM EDTA overnight at 4°C. Subsequently, the GST tag was removed by gel filtration on a S200 Sepharose column in the same buffer by using an Äkta purifier system (GE Healthcare Europe, Munich, Germany). Cortactin-containing fractions were pooled and dialyzed against 25 mM Tris buffer, pH 7.5, containing 150 mM NaCl, and 1 mM DTT. Protein concentration was calculated from the predicted extinction coefficient (Vector NTI software; Invitrogen, Karlsruhe, Germany). Constitutively active Rac1 was also recombinantly expressed as a GST-fusion and cleaved and purified as described previously (Steffen *et al.*, 2004).

Plasmids and Generation of Targeting Constructs

EGFP- β -actin and EGFP-C1 were purchased from Clontech (Mountain View, CA). Constructs driving expression of EGFP-tagged cortactin and myc-tagged L61-Rac1 (pRK5-myc-Rac1Q61L) or L61-Cdc42 (pRK5-myc-Cdc42Q61L) were kindly provided by Ed Schuurung (University Medical Center Groningen, Groningen, The Netherlands) (van Rossum *et al.*, 2003) and Laura M. Machesky (Cancer Research UK Beatson Institute for Cancer Research, Glasgow, United Kingdom), respectively. EGFP-ArpC5B (EGFP-p16B) was as described previously (Rottner *et al.*, 2006), mCherry-clathrin was generated by exchanging EGFP in an EGFP-clathrin construct, a gift from Dr. Ernst Ungewickell (Hannover Medical School, Hannover, Germany) for mCherry, kindly provided by Dr. Roger Tsien (Stanford University, Stanford, CA). For generation of EGFP-tagged, constitutively active Rac1 (Q61L) or Cdc42 (Q61L), the human cDNAs harboring the respective mutations and lacking ATG were excised from pRK5 vectors (see above), and each was fused into pEGFP-C1 (Clontech). Full-length, murine cortactin (*Ctnn*, Ensembl accession no. ENSMUSG00000031078) and the N-terminal fragment (residues 1-146; Ctnn-NT) were generated by polymerase chain reaction (PCR) amplifications using primer pairs F1, 5'-GGCGAATTCTGATGTGGAAAGCCTCTGC-3' and R1, 5'-CCGCTCGAGTCGACCCGCGAACGTGGCGA-3' and F1, R2, 5'-GGTGGATCCTTCTGGGAG GCATGCTTC-3', respectively. For generation of GST-tagged full-length cortactin, the PCR-amplified fragment was subcloned into EcoRI and XbaI of pGEX-6P2 vector (GE Healthcare Europe). EGFP-tagged N-terminal cortactin was made by subcloning Ctnn-NT into EcoRI and BamHI sites of pEGFP-N1 vector (Clontech).

For generation of the targeting constructs, a 7-kb genomic DNA fragment carrying cortactin gene exon 6-9 was used. A synthetic linker containing a *loxP* site was inserted into a HindIII site upstream of exon 7. The neomycin (Neo)/puromycin (Puro) cassette containing the second *loxP* site and two flanking *flp* sites were inserted into the *StuI* site downstream of exon 7. The completed DNA fragments containing the 7-kb cortactin genomic DNA se-

Figure 1. Sequential conditional targeting of both cortactin alleles in murine ES cells. (A) Targeting strategy for cortactin allele. Two targeting constructs carrying either a neomycin (harboring an internal BglII site) or a puromycin (harboring an internal BamHI site) resistance cassette are depicted to target the wild-type cortactin allele (*Cttn*) in two sequential targeting experiments in murine ES cells. (B) Genomic Southern on ES cells carrying two wild-type (wt/wt) cortactin alleles, one wild-type and one floxed (harboring the neomycin cassette) cortactin allele [wt/f^(Neo)], and two floxed [f^(Neo)/f^(Puro)] alleles (1 allele harboring a neomycin and the other allele a puromycin cassette). Genomic DNA extracted from each sample was digested with either BamHI alone, or BamHI together with BglII, and hybridized to the Southern probe (corresponding to genomic DNA sequence outside the targeting vector) as shown in A. Note that only the Puro floxed [f^(Puro)] allele produced a lower hybridization band with BamHI digest alone, whereas both Neo [f^(Neo)] and Puro [f^(Puro)] floxed alleles resulted in a lower hybridization band when double digested with BamHI and BglII. (C) PCR genotyping of ES cells carrying the wild-type, single-targeted or double-targeted alleles. The top and bottom bands indicate the presence of floxed alleles (Neo or Puro) and the wild-type allele, respectively.

quence, the *loxP* and *flp* sites with either Neo or Puro cassettes were subcloned into the pPNT targeting vector backbone (Tybulewicz *et al.*, 1991) to generate ptCttn-Neo and ptCttn-Puro, respectively (Figure 1A). The integrity of the targeting constructs was confirmed both by restriction digests and sequencing of exons. Reverse transcription (RT)-PCR detection of HS1 (*Hcls1*, Ensembl accession no. ENSMUSG00000022831) was performed using the following primers: HS1fw, 5'-GGCTGAAGGCAAAATTTGAG-3' and HS1rv, 5'-TCTCCTCTGCATCCTCATC-3'. Primers specific for SPIN90 (*Nckip5d*, Ensembl accession no. ENSMUSG00000032598) were as follows: SPfw, 5'-CGAGGATCCATGTACCGCGCTGTA-3' and SPrv, 5'-GCAGTCGACCTGGGAACCTCTCCA-3'.

Generation of *Cttn*^{fl/fl} Embryonic Fibroblast Cells

ES cells (line IGD3.2) (Hitz *et al.*, 2007) were modified by two rounds of homologous recombination by using ptCttn-Neo and -Puro constructs according to standard procedures. Double-targeted *Cttn*^{fl/fl} ES (F1) cells were differentiated in vivo by injection into C57BL/6J host blastocysts, which were then transferred into pseudopregnant females. Targeted primary fibroblasts were obtained from E 14.5 chimeric embryos and enriched by double selection with neomycin (500 μ g/ml) and puromycin (5 μ g/ml). Double-targeted primary fibroblasts were immortalized by retroviral transduction of SV40 LT antigen. Clonal *Cttn* del/del cell lines were obtained after adenoviral transduction of Cre recombinase (kindly provided by Cord Brakebusch, University of Copenhagen, Copenhagen, Denmark).

Genotyping

Genomic DNA was prepared from cultured cell samples as described previously (DeChiara, 2001). Genotyping for the floxed (fl) and wild-type (wt) *Cttn* alleles was done with the following set of PCR primers: forward, fS, 5'-agggctgaccatcatgtcc-3' and reverse, fA, 5'-CCCGATTCGACGCATCGCC-3', specific for the wild-type *Cttn* locus upstream of exon 7. PCR products

generated were ~150 bp for the wt allele and ~250 bp for the fl allele (Figure 1C). Cre deletion (which removes exon 7) of the *Cttn* fl allele results in an ~500-bp PCR product being generated using the same forward primer as described above, fS, and a reverse primer (dA, 5'-ggtggtggtggaatgtgtg-3') specific for the neomycin cassette. In the presence of the *Cttn* fl allele, an ~1.2-kb PCR product is generated (Figure 1C). Southern blot analysis of genomic DNA was performed as described previously (DeChiara, 2001). For the screening of ES cell clones, an upstream ~1-kb fragment lying beyond the regions of *Cttn* that had been cloned into the targeting vectors was used as probe for hybridization. Genomic DNA was digested with BamHI and BamHI + BglII for the screening of ptCttn-Neo and ptCttn-Puro targeted clones, respectively. For the detection of Cre-deleted fibroblast clones, genomic DNA was digested with HindIII and hybridized with the 1-kb probe mentioned above.

Cell Culture and Transfection

Puromycin-resistant primary mouse embryonic feeder (MEF) cells (4D) were purchased from Open Biosystems (Huntsville, AL). Feeder and embryonic fibroblast cells were maintained in DMEM (Invitrogen) containing 10% fetal calf serum (Sigma Chemie) and 2 mM glutamine at 37°C in the presence of 5% CO₂. Fibroblast cells were transfected using Metafectene Pro (Biontex, Munich, Germany) or FuGENE HD (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. One day after transfection, cells were either fixed and processed for immunolabeling or examined live by using an inverted microscope as described below.

Fluorescence Microscopy, Assessment of Cell Morphology, and Data Analysis

Immunolabeling experiments were performed essentially as described previously (Lommel *et al.*, 2001; Steffen *et al.*, 2004). In brief, cells grown on

fibronectin were fixed with prewarmed formaldehyde (4%) in PBS for 20 min, extracted with 0.1% Triton X-100 (1 min), and stained with the indicated antibodies, phalloidin, or a combination. Secondary reagents were Alexa dye-labeled (Alexa 350, 488, or 594) goat anti-mouse or anti-rabbit antibodies (Invitrogen). For quantification of PDGF-induced peripheral ruffling and focal adhesion reorganization, cells were seeded subconfluently onto fibronectin-coated glass coverslips, starved, and treated with DMEM alone or DMEM containing 10 ng/ml PDGF (PDGF-BB; Sigma Chemie) for 10 min, fixed as described above, extracted with a mixture of 0.1% Triton X-100 and 4% formaldehyde for 1 min, and stained for vinculin and the actin cytoskeleton with phalloidin. Peripheral ruffling was classified essentially as described previously (Bosse *et al.*, 2007). In addition, cells were categorized according to the prominence of vinculin-positive adhesion sites as indicated. PDGF-induced dorsal ruffling was induced and quantified essentially as described above, except that cells were plated onto uncoated coverslips and treated for 5 min with DMEM or DMEM containing PDGF. Preparations were analyzed on an inverted microscope (Axiovert 100TV; Carl Zeiss, Jena, Germany) by using a 63 $\times$ /1.4 or 100 $\times$ /1.4 plan-apochromatic objectives and equipped for epifluorescence as described previously (Steffen *et al.*, 2004). Images were acquired with a back-illuminated, cooled charge-coupled-device camera (TECCD 800PB; Princeton Scientific Instruments, Princeton, NJ) driven by IPLab software (Scanalytics, Fairfax, VA). Data and images were processed using Excel 9.0 (Microsoft, Redmond, WA) and Adobe Photoshop 7.0/CS software (Adobe Systems, Mountain View, CA).

Video- and Electron Microscopy, and Microinjection

High-magnification video microscopy in combination with or without microinjection was performed with cells mounted in an open, heated chamber (Warner Instruments, Reading, United Kingdom) at 37°C on an inverted microscope (Axiovert 100TV; Carl Zeiss) essentially as described previously (Steffen *et al.*, 2004). Control or cortactin KO fibroblasts with or without expressing EGFP-actin were recorded before and immediately after microinjection of constitutively active Rac1 (L61-Rac1; 2.5 mg/ml) by using phase contrast optics, epifluorescence, or both. Supplemental movies were assembled using NIH Image (<http://rsb.info.nih.gov/ni-image/>). Cell areas before and after Rac1 microinjection were measured using CellProfiler (<http://www.cellprofiler.org/>). Fluorescent recovery after photobleaching (FRAP) experiments were performed on a dual-headed, confocal microscope (FluoView 1000; Olympus, Hamburg, Germany) with cells cotransfected (overnight) with myc-tagged L61-Rac1 and EGFP-actin or EGFP-p16B at a ratio of 2:1, and analyzed as described previously (Lai *et al.*, 2008).

Wound-healing and random cell migration assays were performed essentially as described previously (Pankov *et al.*, 2005; Kopecki *et al.*, 2007) by using an AxioCam MRm camera (Carl Zeiss) on an Axiovert 200 automatic microscope equipped with closed heating and CO₂ perfusion devices. Maximal wound closure rates corresponded to normalized slopes of linear equations derived from regression analyses of the area over time plots shown in Figure 11B. Average wound closure rates were determined by dividing closed areas by required time. For reconstitution of the wound-healing defect, cortactin-deficient cells were transfected either with EGFP alone, with EGFP-L61-Rac1 or with EGFP-L61-Cdc42, and fluorescence-activated cell sorting (FACS) sorted to enrich for EGFP-expressing cells. Wound-healing assays were performed in 12-well plate holes 2 d after seeding of FACS-enriched cells. Data analysis was done using ImageJ (<http://rsb.info.nih.gov/ij/>) and CellProfiler. Total internal reflection fluorescence (TIRF) microscopy and quantification of actin assembly events accompanying clathrin-mediated endocytosis were done as described previously (Benesch *et al.*, 2005).

For electron microscopy, cells were plated on glass coverslips coated with Formvar and 50 μ g/ml fibronectin (Roche Diagnostics), extracted for 1 min with a mixture of 0.5% Triton X-100 and 0.25% glutaraldehyde in cytoskeleton buffer [CB: 10 mM 2-(N-morpholino)ethanesulfonic acid, pH: 6.1, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, and 5 mM MgCl₂] containing 1 μ g/ml phalloidin, fixed for 15 min with 2% glutaraldehyde in CB with 1 μ g/ml phalloidin, and processed for electron microscopy as described previously (Auinger and Small, 2008). Samples were negatively stained with sodium silicotungstate (2%; Agar Scientific, Stansted, Essex, United Kingdom) and aurothioglucose (1%; Wako Chemicals, Düsseldorf, Germany) at pH 7.0. Cells were observed and imaged immediately in an electron microscope (FEI Morgagni, Eindhoven, The Netherlands).

GTPase Activation Assays

Rho-GTPase activities were assessed using absorbance-based G-Lisa kits (individual assays for Cdc42, Rac, and RhoA; Cytoskeleton) according to the manufacturer's protocols. Expression levels of examined Rho-GTPases were independent of cortactin genotype (our unpublished data).

Confirmatory pull-down assays shown in Supplemental Material determining constitutive Cdc42 or Rac activities or PDGF-induced Rac activation were done as described previously (Bosse *et al.*, 2007). Pull-down of active RhoA was done using a commercial activation kit based on the Rho-binding domain (RBD) of rhothekin (Cytoskeleton). Band intensities of active GTPase from at least four independent experiments were each normalized to input (total GTPase), averaged, and expressed relative to levels in respective controls.

Figure 2. Cortactin disruption and expression of regulators of the actin cytoskeleton or endocytosis. (A) Genomic Southern hybridization of immortalized fibroblast clones before and after Cre-mediated deletion. Genomic DNA was digested with HindIII and hybridized to a radioactively labeled 1-kb cortactin probe. Black, white, and gray arrowheads indicate wild-type cortactin allele, Cre-deleted cortactin allele and Neo- or Puro-targeted alleles, respectively. Lane 1, wild-type cells; lanes 2–6, individual clones isolated upon Cre-recombinase-mediated cortactin gene deletion; lane 7, population pooled from clones 2–6; lane 8, double cortactin allele-targeted ES cell clone (genotype flox/flox); and lane 9, single cortactin allele-targeted ES cell clone (genotype flox/wt). (B) PCR genotyping using primers flanking the floxed cortactin exon 7. Top and bottom bands show the presence of floxed/wild-type and deleted alleles, respectively. Lanes are as described in A. (C) PCR genotyping using primers specific for cortactin exon 7. A PCR product indicates the presence of either wild-type or floxed allele. Lanes are as described in A. (D) RT-PCR detection of cortactin mRNA. Top, middle and bottom panels refer to PCR reactions with primers specific for cortactin N-terminal and C-terminal parts, and SPIN90 (loading control), respectively. Lanes 1–7 are as described in A. (E) Immunoblotting with cortactin antibody 4F-11 (top) compared with anti- α -tubulin antibody (bottom). Lanes 1–7 are as described in A. (F) Immunoblotting of control (fl/fl) and cortactin KO fibroblast cells with actin and endocytic regulators as indicated (Cla-HC, clathrin heavy chain). Anti- α -tubulin was used as loading control.

Constitutive Ras activities in control and cortactin-deficient cells were determined indirectly through mitogen-activated protein/ERK phosphorylation relative to input (α -tubulin).

¹²⁵I-Epidermal Growth Factor (EGF) Internalization Assays and Quantification of Surface EGF Receptor (EGFR) and EGFR Degradation

Experiments were essentially as described previously (Haglund *et al.*, 2003; Sigismund *et al.*, 2005). In brief, cells were seeded in triplicates for each time point (plus 1 well for assessment of nonspecific binding) into 24-well plates at a density so that the cells would reach 90% confluence the following day. The next day, cells were serum starved for at least 4 h in binding buffer (DMEM, 0.1% bovine serum albumin, and 20 mM HEPES, pH 7.5) and then incubated at 37°C in the presence of 1 ng/ml ¹²⁵I-EGF (GE Healthcare) in 300 μ l of binding buffer. After different times (2, 4, 6, and 8 min), cells were put on ice, washed twice in cold PBS, and then incubated for 5 min at 4°C in 300 μ l of acidic wash solution (2 M acetic acid and 0.5 M NaCl, pH 2.5). Then, the solution was removed and its radioactivity was measured. This sample represents the amount of receptor-bound ¹²⁵I-EGF at the cell surface. Internalized ¹²⁵I-EGF was determined by cell lysis with 300 μ l of 1 M NaOH and mea-

Figure 3. Cortactin is not essential for lamellipodium protrusion and stress fiber formation. (A) Morphology of control (fl/fl) and cortactin KO fibroblast cells grown on plastic cell culture dishes. Bar, 50 μ m. (B) Phalloidin (phall) staining of fixed control and KO cells on fibronectin-coated glass coverslips. Arrows and arrowheads mark stress fibers and lamellipodia, respectively. Bar, 10 μ m. (C) Immunolabeling with monoclonal anti-cortactin antibodies (clone 289) confirming loss of specific labeling in KO cells. (D) Electron microscopy of negatively stained whole-mount cytoskeletons of control and KO cells showing actin filament networks at the leading edge (cell edges indicated by black arrows). Bar, 50 nm.

surement of radioactivity in the lysate. Nonspecific binding was measured at each time point in the presence of excess, nonradioactive EGF (200 $\times$). After correction for nonspecific binding, the rate of internalization was expressed as the ratio between internalized and surface-bound radioactivity.

The number of EGF binding sites at the cell surface was determined by cell starvation as described above and incubation at 4 $^{\circ}$ C for 6 h with 100 ng/ml 125 I-EGF, 10 ng/ml 125 I-EGF (GE Healthcare), and 90 ng/ml cold EGF (Serochemicals, Norcross, GA). Cells were then washed three times with cold PBS and lysed in 1 M NaOH. The radioactivity measured in the lysate represents the amount of EGFR at the membrane. Nonspecific binding was determined in the presence of excess, nonradioactive EGF (300 $\times$) subtracted from total

Figure 4. Rac1-induced protrusion in the presence and absence of cortactin. (A) Representative individual frames from epifluorescence time-lapse movies of EGFP-actin-expressing control and KO cells before and after microinjection with constitutively active Rac1 (injection at time 0). Time is in minutes and seconds. White arrows highlight lamellipodia induced by this treatment in both cell types. Bar, 10 μ m. (B) Quantification of cell area increase observed within 7.5 min after Rac injection. Data represent means $\pm$ SEM.

radioactivity. EGF binding sites of wild-type and cortactin $-/-$ MEF cells are expressed as receptors per cell.

EGFR degradation was assessed upon EGF treatment (100 ng/ml) for different time points as indicated. Densitometric analyses were aided by ImageJ software. Background-corrected EGFR intensities were normalized to vinculin intensities for each time point and expressed as percentage of the initial amount of EGFR at the cell surface.

RESULTS

Generation of Cortactin KO Fibroblast Cells

To investigate cortactin loss of function, we decided to use a conditional genetic knockout system. Two targeting constructs, harboring either a neomycin (ptCttn-Neo) or a puromycin resistance cassette (ptCttn-Puro), were used to sequentially replace in embryonic stem (ES) cells both wt alleles with mutated alleles harboring *loxP*-sites for recombination (fl; see Figure 1A for targeting strategy). Genomic Southern hybridization and PCR confirmed the presence of the corresponding alleles (Figure 1, B and C) in double-targeted ES cells (fl/fl). Cortactin^{fl/fl} ES cells were subsequently differentiated into fibroblasts in vivo by isolation from chimeric day 14 embryos upon blastocyst injection and transfer. Cortactin^{wt/wt} cells were successfully removed by a 14-d treatment with G418 and puromycin, as confirmed by

Figure 5. Actin network turnover in lamellipodia of control and cortactin KO cells. (A) Representative examples of control (fl/fl) and cortactin-deficient (KO) fibroblasts coexpressing myc-tagged L61-Rac1 and EGFP-actin, and subjected to FRAP experiments in the lamellipodium. Note actin recovery from the lamellipodium front, as expected (Wang, 1985; Lai *et al.*, 2008), in both cell types. Time is in seconds; bar, 2 μm . (B) Quantification of the rate of rearward actin movement based on FRAP experiments as shown in A. All data are means $\pm$ SEM (error bars). Acceleration of actin flow in the absence of cortactin was confirmed to be statistically significant (*) by one-sided two-sample *t* test ($p \leq 0.023$). (C) Lamellipodial actin recovery as assessed by FRAP for a lamellipodial width of 1.6 μm in control and cortactin null cells as indicated. Data are means and SEM measured for each time point and cell type as indicated ($n = 4$). Linear curves correspond to best fits of means (black and red lines for cortactin KO and control cells, respectively). Fitted data followed equation: $y = a(1 - \exp(-bx))$, with $a = 1.0408$ and $b = 0.033$ in fl/fl, and $a = 1.1013$ and $b = 0.044$ in KO cells. $t_{1/2}$ values were calculated by solving the corresponding equations with a fluorescence intensity of 50% of the maximum recovery value derived from each fitted curve. (D) Representative examples of control (fl/fl) and cortactin-deficient (KO) fibroblasts coexpressing myc-tagged L61-Rac1 and the Arp2/3-complex subunit p16B, and subjected to FRAP experiments in the lamellipodium. p16B fluorescence recovers from the lamellipodium front as described in both cell types. (E) Quantification of rearward movement of p16B as shown for actin above. All data are means $\pm$ SEM (error bars). Increased flow in cortactin null cells was statistically significant, as confirmed by one-sided two-sample *t* test ($p \leq 0.046$).

the absence of the wt allele by using PCR (data not shown). Primary cortactin^{fl/fl} fibroblast cells were immortalized and infected with adenovirus-*Cre* (Anton and Graham, 1995) to mediate site-specific recombination of the mutated allele to obtain individual and pooled (from five individual clonal fibroblast lines) cortactin^{del/del} fibroblasts. PCR and genomic Southern confirmed the deleted genotypes of each line individually and of the pooled population (Figure 2A). Successful *Cre* recombinase-mediated deletion of exon 7 in the cortactin gene was expected to cause a stop in the reading frame resulting in a null allele. Indeed, no cortactin message was detectable by PCR, neither with primers complementary to regions encoding the amino-terminal nor carboxy-terminal end of the protein, and we were unable to detect cortactin by Western blotting (Figure 2, B–E, and Supplemental Figure S1). To minimize potential clonal variations, all subsequent experiments were performed with a mixed population of parental cortactin^{fl/fl} cells and pooled cortactin^{del/del} (KO) clones.

Western blot analysis of total cell extracts from cortactin KO and control cells revealed no obvious changes in the expression levels of various prominent actin regulators and endocytic molecules (Figure 2F). Furthermore, no HS1 message or protein, neither in control and nor cortactin KO fibroblast cells was detected (Supplemental Figure 2).

Cortactin Is Not Essential for Arp2/3-Complex-dependent Actin Assembly in the Lamellipodium

Cortactin has been implicated in driving lamellipodium protrusion and membrane ruffling, due to the dependence of these structures on Arp2/3-complex-mediated actin assembly (Goley and Welch, 2006; Steffen *et al.*, 2006). Therefore, lamellipodium protrusion and membrane ruffling were examined in various conditions and by using several methods. Interestingly, no gross morphological changes were initially observed between cortactin KO and control cells on plastic dishes (Figure 3A). We thus explored the actin cytoskeleton in subconfluent cells growing on different substrates such as fibronectin. In the absence of cortactin (Figure 3C), the actin cytoskeleton seemed to be largely unchanged, with cortactin knock-out cells forming lamellipodia and ruffles, filopodia (data not shown), as well as focal adhesions and stress fibers (Figure 3B; also see Figure 8A). Moreover, there was no discernible difference compared with control cells in the ultrastructure of the actin filament network in the lamellipodia of cortactin-deficient cells (Figure 3D). These results suggested that cortactin-deficient cells were able in principle to form lamellipodia. To assess potential quantitative changes in lamellipodia protrusion in the absence of cortactin, we first performed video microscopy before and after microinjection of constitutively active Rac1. Rac1 injection caused a prominent and abrupt induction of smooth, peripheral lamellipodia in both control and knockout cells (see Supplemental Video 1), demonstrating that cortactin is dispensable for Rac-induced protrusion. Using cells ectopically expressing EGFP-tagged actin (Figure 4A and Supplemental Video 2), we assayed lamellipodial actin polymerization *in vivo*. First, no significant differences were observed in the increase in cell area caused by Rac injection between control and knockout cells (Figure 4B), suggesting that Rac-induced actin reorganization was unimpaired by cortactin deficiency. The efficiency of Rac-induced actin assembly in lamellipodia was directly measured by FRAP in cells coexpressing EGFP-actin and constitutively active Rac1 (Figure 5A and Supplemental Video 3). Actin assembly at the lamellipodium tip directly correlates with the rearward movement of the bleached region (Lai *et al.*, 2008). Our FRAP measurements

Figure 6. Actin recruitment to cccps in the absence of cortactin. (A) Representative TIRF images of control (fl/fl) and KO cells cotransfected with EGFP-actin (actin) and mCherry-clathrin (cla) showing cointernalization of actin and clathrin. Red and white arrows mark cointernalizing pits. Bar, 1 μ m. (B) Cointernalization of Arp2/3-complex and clathrin in both control and cortactin KO cells as visualized by cotransfection with EGFP-ArpC5B (p16B) and mCherry-clathrin (cla). Arrows and bar are as described in A. (C) Quantification of actin recruitment to internalizing cccps. All data are means $\pm$ SEM. At least 240 individual pits and 16 cells were analyzed for each cell type.

indicated that actin assembly in the lamellipodium was modestly increased in the absence of cortactin (Figure 5B), resulting in a significant reduction in the half-time of lamellipodial actin recovery in cortactin knockout cells compared with cortactin-expressing controls (Figure 5C; $t_{1/2}$ of 14.9 and 19.4 in KO and control, respectively). Although a molecular explanation for the observed increase in actin rearward flow in cortactin null cells is lacking, these data demonstrate active lamellipodial actin polymerization in both the presence and absence of cortactin. Consistent with this and the view that Arp2/3-complex is essential for Rac-induced, lamellipodial actin assembly (Steffen *et al.*, 2006; Nicholson-Dykstra and

Figure 7. Cortactin is not required for clathrin-mediated endocytosis. (A) Kinetics of EGF internalization in control (fl/fl) and cortactin KO cells treated at the indicated time points with 1 ng/ml 125 I-EGF. Data are means $\pm$ SEM (error bars) of two independent experiments both performed in triplicate. The internalization constants (K_e) are indicated in the panel. (B) EGFR numbers/cell measured as described in *Materials and Methods*. Data are means $\pm$ SEM (error bars) of two independent experiments both performed in triplicate. (C) Western blot analysis of EGFR degradation as well as Akt and ERK activation (pAkt and pERK) in control and cortactin knockout cells treated at the indicated time points with 100 ng/ml EGF. Total Akt, ERK, vinculin, and cortactin were included in the panel as blot normalization. Blots are representative of three independent experiments. (D) Densitometric analysis of EGFR degradation performed as described in *Materials and Methods*. Data are means $\pm$ SEM (error bars) of two independent experiments.

Higgs, 2008), Arp2/3-complex uniformly incorporated into the lamellipodia of both control and cortactin-deficient cells (our unpublished data).

Furthermore, FRAP experiments with cells coexpressing constitutively active Rac1 and the Arp2/3-complex subunit p16B revealed a similar increase of Arp2/3-complex rearward flow rate in the absence of cortactin (Figure 5, D and E, and Supplemental Video 4). This was not unexpected if assuming identical lamellipodial turnover for actin and Arp2/3-complex, which is incorporated into the network at the lamellipodium tip (Figure 5D; Lai *et al.*, 2008). The additional genotype-independent acceleration of network flow rates in p16B compared with actin expressors (Figure 5, B and E) is not due to differential behavior of actin and Arp2/3-complex in individual cells, as proposed previously by others (Cai *et al.*, 2008), because simultaneous bleaching of both components in B16-F1 lamellipodia confirmed identical flow rates (our unpublished data).

Cortactin Is Not Required for Clathrin-mediated Endocytosis

Cortactin has also been shown to accumulate at internalizing clathrin-coated pits (ccps) (Cao *et al.*, 2003; Merrifield *et al.*, 2005; Zhu *et al.*, 2005), suggestive of a role in Arp2/3-complex activation at these sites. We first tested whether both actin and Arp2/3 recruitment to ccps requires cortactin. Cortactin KO and control fibroblast cells were cotransfected with EGFP-actin and mCherry-clathrin and then examined using TIRF microscopy. Interestingly, actin accumulation at ccps was robust in both control and cortactin KO cells (Fig-

ure 6A). Coexpression of EGFP-tagged p16B with mCherry-clathrin revealed that cortactin removal also had no effect on the accumulation of Arp2/3-complex at ccps (Figure 6B). To exclude potential quantitative differences in actin assembly frequencies accompanying clathrin-mediated endocytosis, as observed previously, for example, upon genetic deletion of N-WASP (Benesch *et al.*, 2005), we counted the average coincidence of actin accumulation with the internalization of individual clathrin-coated structures, which was $\sim 80\%$ in both the presence and absence of cortactin (Figure 6C). These results demonstrated that cortactin is not required for actin polymerization accompanying clathrin-mediated endocytosis. To explore a potential functional role for cortactin in this process, independently of the promotion of actin assembly, we initially looked at internalization of rhodamine-labeled EGF or transferrin, the latter being strictly clathrin-dependent. No obvious principal defects in ligand internalization could be detected (our unpublished data). To test for potential quantitative differences of receptor-mediated endocytosis, the initial rate of EGF internalization was measured using iodinated ligand, low doses (1 ng/ml) of which induce internalization predominantly through the clathrin route (Sigismund *et al.*, 2005). However, cortactin deficiency did not affect the efficiency of EGF internalization (Figure 7A). Importantly, these experiments were performed at subsaturating concentrations of the ligand in cortactin-expressing control cells, because cell surface receptor levels were strongly increased in cortactin null cells (Figure 7B). The precise reasons for this are unknown, although no significant defects in EGFR degradation were observed in the ab-

sence of cortactin (Figure 7, C and D). Finally, EGF-induced signal transduction seemed intact in cortactin

null cells, as exemplified by ERK/mitogen-activated protein kinase and Akt phosphorylation (Figure 7C).

Figure 8. Cortactin deficiency abrogates PDGF-induced actin reorganization. (A) Effect of PDGF treatment on cell morphology; cells were stained for the actin cytoskeleton (phall) and for focal adhesions with anti-vinculin antibodies. Arrows and arrowheads mark membrane ruffles and focal adhesions, respectively. Bars, 10 μ m. Note induction of ruffles and concomitant reduction of focal adhesions in control, but not in cortactin KO cells. (B) Quantification of PDGF-treated control (fl/fl) and KO cells capable of forming membrane ruffles. All data are means $\pm$ SEM (error bars). Two-sample two-sided *t* test, $p \leq 0.003$, ~300 cells from three independent experiments categorized for each cell type. (C) Quantification of PDGF-induced focal adhesion disassembly. All data are means $\pm$ SEM (error bars). Two-sided two-sample *t* test, $p \leq 0.025$, >360 cells from three independent experiments categorized for each cell type.

Figure 9. PDGF-induced Rac activation and constitutive activities of selected Rho GTPases. (A) Determination of active Rac levels (Rac-GTP) in starved control and cortactin knockout cells treated with DMEM alone or DMEM containing 10 ng/ml PDGF (10 min) using G-LISA assay. Data are means $\pm$ SEM (error bars) and were statistically compared using two-sample two-sided *t* test. (B–D) Constitutive activities of selected Rho-GTPases in control (fl/fl) and cortactin KO cells as indicated (G-LISA), all data are means $\pm$ SEM (error bars). Reduction of Cdc42 activity in cortactin-deficient cells was confirmed statistically significant using two-sided two-sample *t* test ($p \leq 0.029$).

Cortactin KO Cells Are Defective in Signaling to Actin Reorganization and Rho-GTPase Activation

Although cortactin knockout cells did not fail to form lamellipodia or membrane ruffles upon microinjection or expression of constitutively active Rac (Figures 4 and 5), defects in actin reorganization were unexpectedly detected when treating cells with growth factors such as PDGF. Lamellipodia and peripheral membrane ruffles, known to be driven by Rac, were readily induced by PDGF in serum-starved control but not cortactin knockout cells (Figure 8A). In addition, stress fibers and focal adhesions were mostly dissolved after PDGF treatment in control cells, presumably due to the antagonism between Rac and Rho signaling observed previously (Rottner *et al.*, 1999; Wildenberg *et al.*, 2006). This response was also less prominent in cortactin knockout cells (Figure 8A). Quantification of cells with ruffles or prominent focal adhesions observed upon PDGF treatment revealed a significant decrease in the ruffling frequency in cells lacking cortactin (Figure 8B; $p \leq 0.003$) and a strong defect in the dissolution of focal adhesions (Figure 8C; $p \leq 0.025$).

Furthermore, and in accordance with previous observations upon RNA interference-mediated cortactin depletion (Boyle *et al.*, 2007), cortactin null fibroblasts showed a severe reduction in PDGF-induced dorsal ruffling (Supplemental Figure 3). All these data indicated that cortactin deficiency might cause defective signaling to Rac activation. This was indeed the case, because PDGF-induced Rac activation as measured by G-LISA assay (see *Materials and Methods*) was suppressed in the absence of cortactin, although it was not abolished (Figure 9A). Similar results were obtained using conventional PAK3-CRIB pull-down (Supplemental Figure 4). Somewhat surprisingly, constitutive Rac-GTP levels of cells growing in serum were not significantly different in control and cortactin-deficient cells. In contrast, constitutive Cdc42 activity was also severely suppressed in cortactin-deficient cells, whereas RhoA or Ras signaling were not (Figure 9 and Supplemental Figure 5). Collectively, these data suggest that defective PDGF-induced actin reorganization in cortactin

null cells is caused, at least in part, by reduced Rac activation, low Cdc42 activity, or both. However, in spite of repeated attempts, we have failed to detect convincing Cdc42 activation effected by PDGF treatment in either cell type (our unpublished data).

Cortactin Deficiency Leads to Impaired Cell Migration

Because cortactin seemed to affect actin reorganization events, at least downstream of PDGF signaling, we also explored potential functions in cell migration. To do this, cells were seeded subconfluently, and the tracks of individually migrating cells analyzed in a computer-aided manner. Interestingly, the speed of randomly migrating cells was reduced in the absence of cortactin. Average velocity (arithmetic mean as calculated from the accumulated distance over time) was $0.61 \mu\text{m}/\text{min}$ (SEM = 0.01; $n = 109$) and $0.49 \mu\text{m}/\text{min}$ (SEM = 0.02; $n = 108$) for control and cortactin KO cells, respectively ($p \leq 0.0001$) (Figure 10A). The slight increase in directionality (fl/fl 0.26, SEM = 0.01 vs. KO, fl/fl 0.29, SEM = 0.01) was not statistically significant (Figure 10B). The defect in migration rate was even more evident when color-coding individual tracks (displayed in so called chemotaxis plots) relative to the arithmetic mean of the migration of both cell types (Figure 10C). This analysis revealed that 39 of 109 tracks of controls cells were slower than average, whereas the number of slower cells was significantly increased in the knockout (80 of 108).

We also performed scratch-wound assays, which confirmed reduced migration efficiency (Figure 11A and Supplemental Video 5). Average recovery curves summarizing wound closure rates for both cell types are shown in Figure 11B. The maximal wound healing rate, as extracted by regression analysis (see *Materials and Methods*) was reduced in cortactin KO cells to 67.7% of the control cell population (Figure 11C), with the difference confirmed to be statistically significant ($p \leq 0.001$). These data showed a marked decrease of cell migration rate, as induced here by scratching a wound into a confluent monolayer, shown previously to be

Figure 10. Cortactin deficiency reduces cell migration. (A) Box and whiskers plots showing migration rates of control (fl/fl) and cortactin KO cells in subconfluent cultures. The line within the box indicates median, the box boundaries contain 50% (25–75%) and the whiskers 80% (10–90%) of all measurements, whereas outlying points are shown as dots. The reduction of migration of KO cells was statistically significant at $p \leq 0.0001$; two-sided two-sample t test. (B) Directionality of control (fl/fl) and KO cells as indicated. Data are displayed as in A. Directionality was calculated by dividing net migration distance by total distance. (C) Chemotaxis plots of control (fl/fl) and KO cells in random migration assay. The position of each cell at time zero was centered at the origin of the grid and the tracks correspond to the movement of individual cells over time. Individual tracks slower or faster than the arithmetic mean of all tracks were displayed in red or black, respectively. The total duration of the movies was 495 min.

accompanied by activation of Rho-GTPases such as Rac (Kraynov *et al.*, 2000). To explore whether this defect might be a direct consequence of reduced Rac activity in the absence of cortactin, as observed after PDGF treatment (see above), knockout cells were subjected to the wound-healing assay after transient transfection with EGFP alone or with EGFP-tagged, constitutively active Rac1 (Figure 11D). Average wound closure rates as assessed in these experiments were again significantly reduced in EGFP-expressing cortactin knockout cells compared with (EGFP-expressing) control cells (fl/fl; $p \leq 0.001$). Interestingly, transient expression of constitutively active EGFP-Rac1 significantly increased the performance of cortactin knockout cells, albeit not to the

level of cortactin-expressing control cells (Figure 11D). In contrast, active EGFP-Rac1 decreased the wound-healing efficiency of cortactin-expressing control cells (fl/fl), demonstrating that the observed beneficial effects of Rac activation for migration are dependent on context and restricted here to cells lacking cortactin. These data also highlight the requirement for precise tuning of Rac activity to promote effective migration. Finally, expression of constitutively active Cdc42 in cortactin null cells restored wound-healing rates even better than Rac1, to almost 95% of control cells (Figure 11D). These results are thus consistent with the observed cooperative functions of Rac and Cdc42 small GTPases in the migration efficiency toward PDGF of primary

Figure 11. Loss of cortactin function reduces wound healing efficiency. (A) Selected frames from wound healing movies for control (fl/fl) and cortactin KO cells. Time is in minutes; bar, 100 μ m. (B) Average wound healing recovery curve for each cell population over time. KO cells take longer to cover the wounded area (i.e., reach 100%). (C) Maximal wound closure rates derived from the individual wound closure curves shown in B (see *Materials and Methods*). Data are means $\pm$ SEM (error bars), and the differences between cell types were confirmed to be statistically different by two-sided two-sample *t* test ($p \leq 0.001$). (D) Average wound closure rates of control (fl/fl) or cortactin knock-out (KO) cells expressing EGFP alone (as control) or EGFP-tagged, constitutively active Rac1 or Cdc42 as indicated. Column colors corresponding to experimental conditions are also given on the right in panel D. All data are means $\pm$ SEM (error bars). Differences between cell types or conditions were confirmed to be statistically significant by two-sided two-sample *t* test. *n* values correspond to number of movies recorded in at least three independent experiments.

fibroblasts (Monypenny *et al.*, 2009), and they strongly suggest that the migration defects in cortactin null cells observed here are due to defective signaling to Rho-GTPase activation rather than to problems in promoting or stabilizing Arp2/3-complex-dependent actin networks below the plasma membrane.

DISCUSSION

We report the generation of murine cell lines lacking a functional cortactin gene. Cre-recombinase-mediated ablation of exon 7 within the cortactin locus in conditionally gene-targeted, immortalized fibroblasts resulted in undetectable levels of both cortactin message and protein. Neither control nor cortactin KO fibroblasts expressed the hematopoietic cortactin homologue HS1, excluding functional compensation by HS1.

To our surprise, cortactin removal failed to severely affect the ability of these cells to form a normal actin cytoskeleton. Most remarkably, lamellipodia, which are thought to require Arp2/3-complex-dependent actin assembly (Goley and Welch, 2006) showed no differences in their ultrastructural organization in the absence of this Arp2/3-complex activator (Figure 3D). In addition, Rac injection induced robust lamellipodium formation, spreading and Arp2/3-complex incorporation in both cell types (Figures 4 and 5).

Cortactin was also shown previously to localize on endosomal vesicles (Kaksonen *et al.*, 2000) and clathrin-coated pits (Cao *et al.*, 2003). In addition, it is capable of interaction

with several proteins implicated in endocytic processes, such as dynamin, N-WASP, cortBP1/SHANK2, or WIP (Du *et al.*, 1998; McNiven *et al.*, 2000; Weaver *et al.*, 2002; Aspenstrom, 2005). Due to its biochemical activity as an Arp2/3-complex activator and the fact that its accumulation at ccps peaks at the time point of vesicle fission off the plasma membrane (Merrifield *et al.*, 2005), cortactin was assumed to induce local actin assembly to facilitate the scission process. Moreover, previous observations made in our laboratory have indicated an essential function in actin assembly at these sites for the Arp2/3-complex, activation of which was only partly mediated by N-WASP (Benesch *et al.*, 2005). It was tempting to speculate therefore that cortactin could drive N-WASP-independent Arp2/3-complex activation during clathrin pit internalization. Indeed, studies using RNA interference, antibody injections, or dominant-negative approaches suggested an important function for cortactin in clathrin-mediated endocytosis (Cao *et al.*, 2003; Zhu *et al.*, 2005, 2007), although contradictory observations were also made (Barroso *et al.*, 2006). The results obtained here demonstrate that cortactin is not required for actin and Arp2/3-complex assembly at clathrin-coated pits, and its removal did not even affect the frequency of actin recruitment to internalizing pit-like structures (Figure 6). Because the efficiency of actin assembly is considered crucial for clathrin-mediated endocytosis (Merrifield *et al.*, 2005; Yasar *et al.*, 2005), these data are consistent with the lack of a phenotype when exploring the efficiency of EGF internalization (Figure 7A). Without excluding potential modulatory functions,

these data reveal a nonessential role of cortactin at these sites. The precise function of cortactin in this context remains to be elucidated, not least, due to the increasing number of interacting partners identified (Ammer and Weed, 2008). For example, an interaction of cortactin with huntingtin-interacting protein 1-related (Hip1R) was recently discovered, and both proteins seemed to inhibit actin assembly by capping actin filament barbed ends *in vitro*. The actin filament capping and nucleating activities of cortactin were proposed to be spatially separated *in vivo*, although to both promote actin assembly at ccps (Le Clainche *et al.*, 2007). Interestingly, Hip1R knockdown was shown previously to generate abnormally large actin tails nucleated at these sites, establishing this protein as a potent negative regulator of actin polymerization during endocytosis (Engqvist-Goldstein *et al.*, 2004). Because these exaggerated structures required cortactin, although it was not essential for the actin assembly at ccps observed here, we propose cortactin to execute fine-tuning, perhaps in association with specific proteins or protein complexes (Le Clainche *et al.*, 2007; Ammer and Weed, 2008), rather than constituting a decisive component of actin assembly accompanying clathrin-mediated endocytosis.

In contrast to the structure and dynamics of constitutive or Rac-induced lamellipodia, the frequency of occurrence of lamellipodia and ruffles, induced, for example, by PDGF treatment, was abrogated by cortactin removal. The majority of cortactin KO cells (>60%) did not form membrane ruffles after 10 min of PDGF treatment, whereas most control cells (~90%) did. The impact of cortactin loss of function on PDGF-induced dorsal ruffling was even more severe (Supplemental Figure 3). Moreover, loss of stress fibers and focal adhesions not unusually observed to accompany the induction of membrane ruffling by growth factor treatment, also occurred much less prominent in cortactin-deficient cells (Figure 8, A and C), suggesting that cortactin removal abrogated the signaling pathway causing these various actin reorganizations observed upon PDGF treatment. This would also be in line with the recently established role in this signaling pathway for cortactin phosphorylation by Abl kinases (Boyle *et al.*, 2007). Because the efficiency of lamellipodial actin assembly and Arp2/3-complex incorporation was not reduced upon injection or expression of constitutively active Rac1, this deficiency likely occurs upstream or at the level of Rho-GTPase activation. Consistently, cortactin-deficient cells displayed a marked decrease in Rac activation effected by PDGF treatment (Figure 9A). Furthermore, cortactin null cells displayed a significant reduction of active Cdc42 (but not Rac, Ras, or RhoA) levels in regular growth conditions. These data suggest that cortactin may operate in signaling to actin polymerization less downstream, and hence more indirectly than previously anticipated, i.e., through contributing to the temporal or spatial regulation of activation of selected Rho GTPases (such as Cdc42 or Rac), and not as an essential activator of Arp2/3-complex (Figure 5). Consistent with such a scenario, both cortactin and HS1 have already been reported to interact with GEFs for Rho-family GTPases (Hou *et al.*, 2003; Gomez *et al.*, 2006). Notably, the recruitment of Fgd1 to the cell periphery by interaction with the SH3 domain of cortactin was proposed to contribute—upon multimerization—to a positive feedback cycle of cortactin-mediated actin assembly (Kim *et al.*, 2004). Although not necessarily restricted to the latter guanine nucleotide exchange factor (GEF), these observations point toward a potential role for cortactin in signal amplification through recruitment—not necessarily activation—of Rho-GTPase GEFs to sites of dynamic actin assembly. A potential function as “localizer” rather than stimulator of efficient

Rho-GTPase activation would explain the absence of protrusion induction and evident Rho-GTPase activation by sole ectopic expression (Lai *et al.*, 2008; our unpublished observations).

As we show, cortactin-deficient cells displayed defects in the efficiency of cell migration, which could be reconstituted in part by ectopic expression of constitutively active Rac1 or Cdc42. Given that EGFP-Rac1/Cdc42-transfected and FACS-sorted cortactin knockout cells had to grow to confluence before the wound-healing assay could be performed, incomplete reconstitution could derive, at least in part, from inhomogeneous expression of the ectopic protein. Nevertheless, these data show that a good fraction of the cell migration defects in cortactin null cells can be explained by inefficient signaling to selected Rho-GTPases, consistent with the observations made upon PDGF stimulation (see above). Although Cdc42 is not essential for cell migration in fibroblasts (Czuchra *et al.*, 2005), our data add to increasing evidence that Cdc42 activity can contribute to migration efficiency in certain conditions or cell types (Yang *et al.*, 2006; Monypenny *et al.*, 2009).

In summary, we show that cortactin deficiency leads to defects in migration and to suppression of peripheral or dorsal ruffling in response to PDGF. Surprisingly, however, no impairment of actin assembly in lamellipodia or ruffles was observed once they had formed, nor in other types of Arp2/3-complex-dependent actin structures, e.g., clathrin-coated pits. From these data, we suggest that cortactin operates in signal transduction upstream of activation of specific Rho-family GTPases, rather than downstream, a novel role that may or may not involve its ability to activate Arp2/3-complex-mediated actin assembly. Future studies will address this issue as well as the molecular link(s) mediating this function.

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