Cortactin Is a Substrate of Activated Cdc42-Associated Kinase 1 (ACK1) during Ligand-induced Epidermal Growth Factor Receptor Downregulation

Laura C. Kelley*, Scott A. Weed*

Department of Neurobiology and Anatomy, Program in Cancer Cell Biology, Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, West Virginia, United States of America

Abstract

Background: Epidermal growth factor receptor (EGFR) internalization following ligand binding controls EGFR downstream pathway signaling activity. Internalized EGFR is poly-ubiquitinated by Cbl to promote lysosome-mediated degradation and signal downregulation. ACK1 is a non-receptor tyrosine kinase that interacts with ubiquitinated EGFR to facilitate EGFR degradation. Dynamic reorganization of the cortical actin cytoskeleton controlled by the actin related protein (Arp)2/3 complex is important in regulating EGFR endocytosis and vesicle trafficking. How ACK1-mediated EGFR internalization cooperates with Arp2/3-based actin dynamics during EGFR downregulation is unclear.

Methodology/Principal Findings: Here we show that ACK1 directly binds and phosphorylates the Arp2/3 regulatory protein cortactin, potentially providing a direct link to Arp2/3-based actin dynamics during EGFR degradation. Co-immunoprecipitation analysis indicates that the cortactin SH3 domain is responsible for binding to ACK1. In vitro kinase assays demonstrate that ACK1 phosphorylates cortactin on key tyrosine residues that create docking sites for adaptor proteins responsible for enhancing Arp2/3 nucleation. Analysis with phosphorylation-specific antibodies determined that EGFR-induced cortactin tyrosine phosphorylation is diminished coincident with EGFR degradation, whereas ERK1/2 cortactin phosphorylation utilized in promoting activation of the Arp2/3 regulator N-WASP is sustained during EGFR downregulation. Cortactin and ACK1 localize to internalized vesicles containing EGF bound to EGFR visualized by confocal microscopy. RNA interference and rescue studies indicate that ACK1 and the cortactin SH3 domain are essential for ligand-mediated EGFR internalization.

Conclusions/Significance: Cortactin is a direct binding partner and novel substrate of ACK1. Tyrosine phosphorylation of cortactin by ACK1 creates an additional means to amplify Arp2/3 dynamics through N-WASP activation, potentially contributing to the overall necessary tensile and/or propulsive forces utilized during EGFR endocytic internalization and trafficking involved in receptor degradation.

Citation: Kelley LC, Weed SA (2012) Cortactin Is a Substrate of Activated Cdc42-Associated Kinase 1 (ACK1) during Ligand-induced Epidermal Growth Factor Receptor Downregulation. PLoS ONE 7(8): e44363. doi:10.1371/journal.pone.0044363

Editor: Neil A. Hotchin, University of Birmingham, United Kingdom

Received July 28, 2011; Accepted August 6, 2012; Published August 30, 2012

Copyright: © 2012 Kelley, Weed. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by United States National Institutes of Health (NIH) Grants DE014364, DE014578 and a subproject of RR16440 to SAW. LCK was supported in part by the West Virginia University Office of Research and Graduate Education. The West Virginia University Microscope Imaging Facility, Mary Babb Randolph Cancer Center is supported by NIH grants P20 RR16440 and P30 RR032136/GM103488. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: sweed@hsc.wvu.edu

† Current address: Biology Department, Duke University, Durham, North Carolina, United States of America.
ACK1 and Cortactin in EGFR Regulation

[11,12]. ACK1 is a multidomain non-receptor tyrosine kinase that contributes to EGFR internalization and subsequent vesicular trafficking [reviewed in [13]]. EGFR stimulation recruits ACK1 to activated EGFR through an ACK1 carboxyl-terminal region highly homologous to the EGFR binding protein Mig6, which in turn activates ACK1 kinase activity [14,15]. ACK1 also interacts with clathrin heavy chain [16,17] and Grb2 [14], providing additional attachment points to the endocytic machinery. While ACK1 is well positioned to govern EGFR CME, studies manipulating ACK1 expression levels to evaluate the impact of ACK1 on EGFR internalization have yielded opposing results. In some cases ACK1 overexpression impairs and knockdown promotes EGFR internalization [18,19]. These findings are in agreement with studies on transferrin receptor CME with the ACK1 splice variant ACK2 [20]. Other work indicates that ACK1 knockdown suppresses EGFR internalization and degradation [15]. This effect was verified by expression of the ACK1 Mig6 domain and with an ACK1 ubiquitin-binding deficient mutant [21,22]. Like EGFR, in some instances ACK1 itself is downregulated following EGF stimulation due to Nedd4 ubiquitination, targeting ACK1 for either conventional proteosomal [21] or lysosomal [23] degradation depending on the specific Nedd4 isoform. Nedd4-1 ubiquitination of ACK1 is required for EGFR degradation, indicating that at least a subset of EGFR/ACK1 complexes are targeted for vesicular-mediated co-destruction. Supporting these biochemical findings, activated ACK1 is enriched in clathrin-coated pits and colocalizes EGFR in early endosomal vesicles following EGFR stimulation [15,17,18], suggesting that ACK1 activity regulates EGFR vesicle trafficking in the clathrin pathway.

In addition to collective tyrosine kinase activity at clathrin coated pits, receptor internalization by CME involves coordinate regulation of the cortical actin cytoskeleton by actin regulatory proteins [reviewed in [24]]. Activation of Arp2/3 complex, a major actin nucleation apparatus, results in dynamic branched actin network formation at the site of clathrin coated pits that contributes to vesicle invagination, scission and intracytoplasmic transport [25]. Arp2/3 complex activity is stimulated by proteins termed nucleation promoting factors (NPFs) [26]. The type I NPFs of the WASp protein family N-WASp and WASH play important roles in governing Arp2/3 actin polymerization that drives CME and vesicle trafficking [27]. In addition to type I NPFs, the type II NPF cortactin plays a direct role in regulating CME and vesicle motility [28,29,30]. Cortactin links the Arp2/3 F-actin network to CME through binding of its carboxyl terminal SH3 domain to the large GTPase dynamin2 [31,32]. Cortactin and dynamin2 are both tyrosine phosphorylated by Src, events that are required for each protein to function during CME [33,34]. In the case of cortactin, tyrosine phosphorylation by Src or Abl-family kinases occurs on three tyrosine residues (Y421, 466 and 486 in rodent forms; Y421, 470 and 486 in human) that create docking sites for the adaptor Nck1, which in turn complexes with N-WASp and WIP to amplify Arp2/3 nucleation and enhance actin polymerization levels [35,36]. In addition to promoting actin nucleation during endocytosis, the cortactin SH3 domain also binds Hip1R to suppress actin dynamics during CME [37], indicating that cortactin serves as an important intersection point in regulating endocytic actin polymerization by phosphorylation -independent and -dependent processes.

EGFR internalization and lysosomal degradation relies in part on direct interactions between the activated receptor and F-actin [38]. Additionally, cortactin modulates EGFR internalization through actin-based interactions with the adaptor protein CD2AP in circular dorsal ruffles, which also require cortactin tyrosine phosphorylation to form [36,39]. While these studies did not evaluate EGFR degradation, subsequent work in tumor cells with amplified cortactin gene levels indicates that cortactin upregulation prevents EGFR degradation [40], although the mechanism for this is unclear. Here we report that cortactin interacts with ACK1 to potentially regulate EGFR degradation. Cortactin is directly phosphorylated by ACK1 and both proteins localize in vesicles with ligand bound EGFR. Downregulation of cortactin tyrosine phosphorylation is coincident with EGFR degradation, while knockdown of ACK1 or cortactin attenuates EGFR downregulation through the cortactin SH3 domain. Our results provide evidence for a novel linkage between ACK1 and cortactin that may play an important role in coupling EGFR to the actin cytoskeleton to facilitate EGFR degradation.

Materials and Methods

Cell Culture and Transfection

COS1 and 293T cells (purchased from the American Type Culture Collection) were maintained in DMEM (Mediatech) containing 10% FBS (HyClone), 1% L-glutamine and 1% penicillin-streptomycin. 1483 and 584 HNSCC cell lines [41,42] were maintained as described [43]. COS1 cells (1x10⁷) were transfected with 5 micrograms of plasmid DNA using SuperFect (Qiagen). 293T, 1483 and 584 cells (3x10⁶) were transfected with 2 micrograms of plasmid DNA or siRNA using the Nucleofector I device (Amaxa Biosystems). Control and human targeted siRNA for ACK1 (5’-AAAGUGUCAGCAGCACGCUA-3’) were purchased from Thermo Scientific. Cortactin siRNA (catalog # SI00300160) was purchased from Qiagen.

Cloning of Murine ACK1

Murine ACK1 (GenBank # AF037260) was cloned using two sets of degenerate oligonucleotide primers (primer 1: 5’-GAT/ C/ACNTGGATGTT/T/C/GGNT-3’; primer 2: 5’-TGGGA/A/GATGTT/T/C/ACNTA/T/C/GG-3’; primer 3: 5’-GGNGG/A/GTGATNGGA/A/GTTNC-3’ and primer 4: 5’-TCNGGA/A/GAANCCCA/A/GCA/A/G/TG-3’). These primers were used in nested PCRs to generate a 450 bp probe from a mouse brain cDNA library (Novagen). This probe was labeled with 32P-dCTP using the RadPrime DNA labeling system (Invitrogen) and used to screen a mouse brain lambda GT10 cDNA library (Stratagene). Positive overlapping clones were identified after three rounds of screening with high stringency conditions. The reconstructed ACK1 sequence consisted of a 1,002 amino acid open reading frame with a polyadenylation tail, but lacked a Kozak consensus sequence with an initiation methionine. The 5’ ACK1 sequence was isolated using a two-step nested PCR approach. The first step used the ACK1 specific primer 5’-ACTGAGATGAGAAGATCGCC-3’ with a T7 terminator primer homologous to the vector sequence. The product from this reaction was used as the template for the second reaction using the ACK1 specific primer 5’-TGTTCTTGATGACATTTGAG-3’ with a primer homologous to the SP6 vector sequence. This reaction generated a 1 kb PCR product containing >600 bp overlap with the original isolated ACK1 cDNA. A Kozak sequence and initiation methionine were identified 53 amino acids upstream from the 5’-end of the original ACK1 clone. Additional PCR was conducted to generate the full-length murine ACK1 coding sequence.

Mammalian Expression Constructs

For generating HA-tagged ACK1 variants, full-length ACK1 cDNA was excised from pBluescript SK+ as a BamHI/EcoRV

PLOS ONE | www.plosone.org 2 August 2012 | Volume 7 | Issue 8 | e44363
fragment and subcloned into BamHI/EcoRV digested pSV containing a 5X HA epitope tag. The kinase null K158R (KR) and Cdc42-binding null H464/H467Q (BD) ACK1 variants were produced by site directed mutagenesis (QuickChange; Stratagene). Myc-tagged ACK1 constructs were produced by BamHI/EcoRI digestion of pSV-HA ACK1 constructs and subcloning the ACK1 fragments into pRK5Myc [44]. Myc-Cdc42, Myc-RhoA, FLAG-cortactin and CMV-Src527F constructs have been described previously [44,45,46,47].

Recombinant Protein Production

The ACK1 coding sequence was PCR amplified as an EcoRI/BglII fragment and subcloned into the baculoviral expression vector pAcHHLT-B (Pharmingen). The kinase null K158R (KR) and Cdc42-binding null H464/H467Q (BD) ACK1 variants were produced by site directed mutagenesis (QuickChange; Stratagene). Myc-tagged ACK1 constructs were produced by BamHI/EcoRI digestion of pSV-HA ACK1 constructs and subcloning the ACK1 fragments into pKK5Myc [44]. Myc-Cdc42, Myc-RhoA, FLAG-cortactin and CMV-Src527F constructs have been described previously [44,45,46,47].

Enzyme kinetics for cortactin phosphorylation by ACK1 were calculated using the predicted molecular weight for ACK1 including the 6X-His tag (119.33 kilodaltons) and autoradiogram band intensities to generate a Lineweaver-Burke plot and the subsequent K_M value.

Antibodies

Anti-ACK1 (clone A-11) and EGFR (clone 1005) were purchased from Santa Cruz. Anti-phosphotyrosine (RC 20) was purchased from BD Transduction. Anti-Cdc42, RhoA, Anti-FLAG (DDDDK tag), anti-Myc epitope tag (4A6) and anti-beta-actin were purchased from EMD Millipore. Anti-cortactin (4F11) and anti-pS418 cortactin were previously described [51,52]. Anti-pY421 cortactin was from Invitrogen. Anti-HA (3F10) was from Roche.

Immunoprecipitation and Western Blotting

For immunoprecipitations, cells were lysed in NP40 buffer (20 millimolar HEPES-KOH, pH 7.8, 50 millimolar KCl, 1 millimolar EDTA and 1% NP40). Five micrograms of primary antibody (anti-cortactin, EGFR, and HA) were incubated with 0.5 milligrams clarified lysates for 2 h at 4°C, then incubated with 40 microliters of Protein A/G beads (Thermo Scientific) for 1 h at 4°C. FLAG immunoprecipitations were preformed with 40 microliters of anti-FLAG M2 affinity gel (Sigma). Immune complexes were collected by centrifugation, washed twice with NP40 Buffer, separated by SDS-PAGE and Western blotted as described [50]. In some cases, cells were serum starved for 16 h and stimulated with 100 nanograms/milliliter EGF (Millipore).
prior to immunoprecipitation and/or Western blotting. Band intensities were quantified using ImageJ.

Confocal Microscopy

Cells were serum-starved for 16 h and stimulated with 100 nanograms/milliter unlabeled EGF (Millipore) or Alexa Fluor-488 conjugated EGF (Invitrogen) for 30 minutes before fixation. Cells were fixed with fresh 4% formaldehyde and permeabilized with 0.4% Triton X-100 in PBS. Primary antibodies listed were diluted with 5% BSA in PBS. Secondary antibodies used were Alexa Fluor 405 goat anti-rabbit and Alexa Fluor 647 goat anti-mouse (Molecular Probes). F-actin was labeled with rhodamine-conjugated phalloidin (Molecular Probes). Cells were mounted in Fluoromount-G (Southern Biotech) and imaged with a Zeiss LSM510 confocal microscope using AIM software (Carl Zeiss MicroImaging). Invadopodia and matrix-degradation assays were performed as previously described [47].

Results

Activation of Cdc42 Enhances Cortactin Tyrosine Phosphorylation through ACK1

Our previous work demonstrated that cortactin tyrosine phosphorylation is regulated in part by the small GTPase Rac1 [50]. To determine if other Rho family members influence cortactin tyrosine phosphorylation, we conducted a series of co-transfection experiments utilizing dominant negative and constitutive active constructs encoding the Rac1-related GTPases RhoA and Cdc42 with FLAG-tagged cortactin and assayed total cortactin tyrosine phosphorylation by Western blotting. Expression of RhoA activation variants caused no detectible changes in cortactin tyrosine phosphorylation (Figure S1). However, co-expression of constitutive active Cdc42 (L61) consistently resulted in six-fold increases in cortactin tyrosine phosphorylation (Figure 1A). Activated Cdc42 is the only small GTPase that directly interacts with the tyrosine kinase ACK1 through a conserved Cdc42/Rac interaction binding (CRIB) motif [53], stimulating ACK1 tyrosine kinase activity [54,55]. To determine if ACK1 was the kinase potentially responsible for cortactin tyrosine phosphorylation downstream of activated Cdc42, we conducted FLAG-cortactin co-transfection experiments with HA-tagged wild-type (WT) or Y421/466/482F (triple tyrosine mutant; TYM) (0.5 micrograms) were incubated in kinase assays without or with 3U of purified Src (left) or with the indicated amounts of purified 6X-His ACK1 (middle and right) in the presence of [gamma-32P]ATP. Reactions were separated by SDS-PAGE and analyzed by autoradiography. Arrows indicate the positions of phosphorylated GST-cortactin and 6X-His ACK1 (left). (C) Determination of the $K_m$ of ACK1 for cortactin. Graphical representation showing ACK1 phosphorylation kinetics for cortactin calculated from the kinase assay in B (right panel). Percent of maximum phosphorylation signal measured by densitometry is represented on the ordinate versus concentration of ACK1 (in micrograms) on the abscissa. The calculated $K_m$ for cortactin phosphorylation is shown. Data in B and C are representative from three independent experiments. (D) ACK1 knockdown reduces cortactin phosphorylation on tyrosine 421. 1483 cells transfected with scrambled control (Ctl) siRNA or ACK1-targeting siRNA were lysed and analyzed by Western blotting for ACK1 knockdown (ACK1) and cortactin pY421 phosphorylation (Cort pY421). Beta actin was blotted to verify equivalent protein loading. Blots are representative of two independent experiments. doi:10.1371/journal.pone.0044363.g002

Figure 2. ACK1 directly phosphorylates cortactin at Y421/466/482. (A) Characterization of purified ACK1. Recombinant 6X-histidine tagged recombiant ACK1 was purified from SF9 cells and 5 micrograms stained with either Coomassie blue (left) or analyzed by Western blotting with anti-ACK antibodies (middle). Kinase activity was assayed by analyzing autophosphorylation (Autophos) using 0.5 micrograms of 6X-His ACK1 incubated with gamma-32P-ATP for 10 min at 30°C. The reaction was evaluated by SDS-PAGE and autoradiography (right). The position of molecular weight standards is shown on the left and 6X-His ACK1 on the right. (B) Identification of cortactin tyrosine residues phosphorylated by ACK1. Purified GST-cortactin murine wild type (WT) or Y421/466/482F (triple tyrosine mutant; TYM) (0.5 micrograms) were incubated in kinase assays without or with 3U of purified Src (left) or with the indicated amounts of purified 6X-His ACK1 (middle and right) in the presence of [gamma-32P]ATP. Reactions were separated by SDS-PAGE and analyzed by autoradiography. Arrows indicate the positions of phosphorylated GST-cortactin and 6X-His ACK1 (left). (C) Determination of the $K_m$ of ACK1 for cortactin. Graphical representation showing ACK1 phosphorylation kinetics for cortactin calculated from the kinase assay in B (right panel). Percent of maximum phosphorylation signal measured by densitometry is represented on the ordinate versus concentration of ACK1 (in micrograms) on the abscissa. The calculated $K_m$ for cortactin phosphorylation is shown. Data in B and C are representative from three independent experiments. (D) ACK1 knockdown reduces cortactin phosphorylation on tyrosine 421. 1483 cells transfected with scrambled control (Ctl) siRNA or ACK1-targeting siRNA were lysed and analyzed by Western blotting for ACK1 knockdown (ACK1) and cortactin pY421 phosphorylation (Cort pY421). Beta actin was blotted to verify equivalent protein loading. Blots are representative of two independent experiments. doi:10.1371/journal.pone.0044363.g002

PLOS ONE | www.plosone.org 4 August 2012 | Volume 7 | Issue 8 | e44363
Cdc42 stimulates cortactin tyrosine phosphorylation through activation of ACK1.

ACK1 Directly Phosphorylates Cortactin on Tyrosines 421/466/482

Phosphorylation of cortactin on tyrosines 421, 466 and 482 by several non-receptor tyrosine kinases indicates that these residues serve as important regulatory targets that mediate signals responsible for governing cortical actin-based processes. To determine if ACK1 directly phosphorylates cortactin on these residues, we generated baculovirus encoding full-length 6X-histidine tagged recombinant ACK1 and purified the kinase from infected SF9 cells. Purified ACK1 migrated at a Mr of 145 kDa by SDS-PAGE, similar to the mobility observed in other cell systems [14,16] and possessed functional kinase activity as determined by autoradiographical analysis of autophosphorylation (Figure 2A).

Incubation of 6X-His ACK1 with GST-cortactin wild type (WT) in kinase reactions containing [gamma-32P]ATP resulted in GST-cortactin phosphorylation similar to levels observed when cortactin was incubated with purified Src (Figure 2B). Incubation of GST-cortactin containing tyrosine to phenylalanine mutations at Y421, Y466 and Y482 (triple tyrosine mutant; TYM) with 6X-His ACK1 nearly abolished cortactin phosphorylation (Figure 2B), with residual phosphorylation of additional sites evident at extended autoradiogram exposure times (data not shown). Increasing amounts of 6X-His ACK1 with fixed amounts of GST-cortactin WT resulted in saturable levels of cortactin phosphorylation (Figure 2B and C), with a calculated Michaelis constant for cortactin phosphorylation equaling ~140.7 nM. To evaluate the level of endogenous phosphorylation of the identified cortactin tyrosine sites by ACK1 in cells, 1483 HNSCC cells were transfected with non-targeting (Ctl) or ACK1-targeted small interfering (si)RNA. Immunoblot analysis indicated an 82% knockdown of ACK1 expression, which corresponded with an 84% decrease in cortactin phosphorylation at tyrosine 421 (Figure 2D). These results collectively indicate that ACK1 directly phosphorylates cortactin primarily at Y421/Y466/Y482 in a manner similar to other cortactin-targeting tyrosine kinases.

The Cortactin SH3 Domain Mediates Binding to ACK1

In order to determine how cortactin interacts with ACK1, 1483 HNSCC cells were transfected with Myc-tagged ACK1 (to enhance ACK1 detection) and assayed for cortactin binding by co-immunoprecipitation. Myc-ACK1 precipitated with endogenous cortactin (Figure 3A), suggesting the two proteins interact. The immunoprecipitation was specific, since beads lacking anti-cortactin antibody did not precipitate Myc-ACK1 (Figure S2). We next cotransfected Myc-ACK1 with FLAG-cortactin deletion

![Figure 3. Identification of the ACK1 binding region in cortactin.](image-url)
constructs in order to map the cortactin subdomains responsible for ACK1 binding. Myc-ACK1 precipitated with FLAG-cortactin full length (FL) and carboxyl terminal (CT) (amino acids 350–546) proteins, but not with a FLAG-cortactin protein containing the amino terminal (NT) Arp2/3 and F-actin binding regions (Figure 3B). These results localize the cortactin binding site for ACK1 to the carboxyl-terminal half of cortactin. The cortactin SH3 domain at the carboxyl-terminus binds to proline-rich segments in several proteins involved in actin regulation and endocytosis [56] while ACK1 contains several proline-rich segments within its carboxyl terminus [53]. To determine if the cortactin SH3 domain binds to ACK1, Myc-ACK1 was cotransfected with a cortactin construct containing a point mutation within the SH3 domain that ablates binding to proline-rich ligands (W525K) [46]. This protein displays retarded electrophoretic mobility in SDS-PAGE, presumably due to altered conformation.

Figure 4. Cortactin localizes with ACK1 in vesicles containing ligand-bound EGFR. (A) 1483 cells serum starved for 16 h were stimulated with 100 nanograms/milliter Alexa Fluor-488 conjugated EGF (green) for 30 min. Cells were fixed and labeled with phalloidin (Actin; pseudocolored white) and anti-EGFR antibodies (pseudocolored red). Cells were evaluated by confocal microscopy and images rotated 45° and 90° as indicated to demonstrate EGF/EGFR colocalization throughout the z-plane. (B) Serum starved (No Tx) 1483 cells were stimulated with FITC-EGF (pseudocolored white) as in (A). Cells were fixed and labeled with anti-ACK1 (green) and cortactin (red) antibodies. Confocal images of labeled EGF in the apical (top) and ventral (bottom) cellular regions are shown. Dashed boxes in the merged images indicate the areas enlarged in the photos to the right. Scale bars, 20 micrometers.
introduced by the point mutation as previously observed [46]. FLAG-cortactin W525K failed to co-precipitate Myc-ACK1 (Figure 3B), indicating that cortactin SH3 domain is responsible for linking cortactin to one or more proline rich sequences in ACK1.

### ACK1 and Cortactin Localize in Vesicles Containing Ligand-bound EGFR

Since ACK1 and cortactin have been individually shown to participate in regulating EGFR surface expression following ligand stimulation [13,18,40], we determined if both proteins localized at sites containing activated EGFR. Initial experiments in 1483 cells stimulated with Alexa Fluor-488 conjugated EGF for 30 min indicated that EGF/EGFR complexes were present throughout the vertical dimension of the cell when analyzed by whole cell confocal analysis, demonstrating internalization of activated EGFR in this system (Figure 4A). Confocal imaging of the apical and basal planes of fluorescently-labeled EGF stimulated 1483 cells co-immunostained with antibodies against ACK1 and cortactin indicated co-localization of ACK1 and cortactin at apical internalization regions as well as in EGF-containing vesicles at the ventral surface of the cell (Figure 4B). These vesicles contained activated EGFR that co-localized with cortactin (Figure S3), collectively demonstrating that cortactin localizes with ACK1-containing vesicles associated with activated EGFR during receptor internalization.

In addition to regulating CME, cortactin tyrosine phosphorylation has a prominent role in governing podosome and invadopodia function in invasive cells [47,57,58]. Myc-ACK1 did not localize to podosomes or invadopodia in invasive Src-transformed HNSCC cells, nor did knockdown of ACK1 expression impact the ability of podosomes/invadopodia to degrade ECM (Figure S4). These results suggest that ACK1 does not participate in cortactin phosphotyrosine-based signaling involved in regulating podosome or invadopodia function.

### Cortactin Phosphorylation is Differentially Regulated during EGFR Degradation

The ability of ACK1 to bind and phosphorylate cortactin, along with the localization of ACK1 and cortactin with EGFR during ligand stimulated receptor internalization, suggests that cortactin tyrosine phosphorylation may be modulated during the internalization process. Additionally, EGFR activation stimulates ERK1/2 activity, which in turn phosphorylates cortactin at S405 and S418 to regulate cortactin SH3 domain binding to N-WASp and other proteins [59,60]. To monitor cortactin tyrosine and serine phosphorylation status during EGFR internalization, Western blotting of whole cell lysates was conducted with phosphorylation-specific antibodies against tyrosine 421 (pY421) and serine 418 (pS418). EGF treatment of starved 1483 cells induced cortactin tyrosine phosphorylation evident at 15 min, which gradually decreased until it was not detectable two h post stimulation (Figure 5A). The reduction of cortactin tyrosine phosphorylation mirrored ligand-induced EGFR downregulation and was not due to decreased cortactin protein levels. EGF-induced ERK1/2 cortactin phosphorylation persisted throughout the entire time course and did not decrease during EGFR degradation (Figure 5B). These results indicate that cortactin tyrosine and serine phosphorylation undergoes disparate regulation during EGFR downregulation.

### The Cortactin SH3 Domain is Required for Ligand-induced EGFR Downregulation

To determine if a potential ACK1/cortactin linkage contributes to EGFR internalization, we conducted siRNA knockdown and rescue experiments of ACK1 and cortactin to evaluate effects on EGFR degradation. 1483 cells treated with scrambled (Ctl Si) siRNA demonstrated complete EGFR degradation at two h when evaluated by immunoprecipitation (Figure 6A). RNAi depletion of ACK1 (ACK1 Si) prevented EGFR downregulation in ligand stimulated cells, indicating that ACK1 plays an essential role in promoting EGFR internalization in 1483 cells. Rescue of ACK1 cells by expression of Myc-ACK1 restored the ability of 1483 cells to degrade EGFR, albeit with delayed degradation kinetics (Figure 6A).

Finally, to determine if cortactin plays a similar role to ACK1 in EGFR downregulation, EGFR degradation was assayed in cortactin knockdown and rescued 1483 cells following EGF treatment. Transfection of cortactin siRNA (CTTN Si) resulted in increased EGFR accumulation in starved cells and slowed EGFR degradation compared with control siRNA treatment (Figure 6B). Transfection of FLAG-cortactin in CTTN Si cells also delayed EGFR degradation compared to control siRNA cells (Figure 6A). This result may be due to the inhibitory effects of cortactin overexpression as previously reported in HNSCC cells [40]. However, rescue of CTTN Si cells with FLAG-cortactin W525K blocked EGFR degradation in a manner similar to that in
stimulated cells with ACK1 knockdown (Figure 6A and B). Overall, this indicates that ACK1 and the cortactin SH3 domain are required for ligand induced EGFR downregulation, potentially through a direct interaction between the two molecules.

**Discussion**

ACK1 has been implicated in multiple aspects of normal cellular and neoplastic processes, including growth, invasion and endocytic regulation of growth factor receptors [13]. Ligand induced downregulation of EGFR is an important regulatory mechanism for controlling EGFR signaling that involves CME of the receptor and ultimate trafficking to lysosomes [2, 61]. Evidence to date indicates that regulated actin polymerization at all points of the internalization pathway is vital in driving the membrane dynamics necessary for EGFR internalization, with cortactin serving as an important scaffold by coupling Arp2/3 activity to cytoplasmic membrane surfaces during internalization [28, 34, 62].

Our present findings provide evidence that ACK1 directly phosphorylates cortactin at tyrosine sites that provide enhanced Arp2/3-based actin polymerization, which may be utilized to facilitate EGFR internalization. ACK1 associates with the cortactin SH3 domain, providing a potentially important interac-

---

**Figure 6. EGFR downregulation requires ACK1 and the cortactin SH3 domain.** (A) 1483 cells were transfected with non-targeting (Ctl) or human-specific ACK1 siRNA (ACK1 Si) for 48 h. Murine Myc-ACK1 was subsequently transfected into ACK depleted cells to rescue ACK1 expression. Cells were serum starved for 16 h and then treated with EGF for the indicated times. Following stimulation, clarified lysates were immunoprecipitated and immunoblotted with anti-EGFR antibodies. Total cell lysates were immunoblotted with anti-ACK1 and anti-actin antibodies. (B) 1483 cells were transfected with a non-targeting (CTL) or cortactin specific siRNA (CTTN Si) for 48 h. Cortactin expression was rescued by transfection with FLAG-cortactin wild type (WT) or with an SH3-null binding mutant (W525K). Cells were serum starved for 16 h prior to EGF stimulation for the indicated times. EGFR was immunoprecipitated and immunoblotted with anti-EGFR antibodies. Total cell lysates were immunoblotted with anti-cortactin to verify knockdown and expression of the FLAG-cortactin rescue constructs. Western blotting with anti-actin antibodies was conducted to verify equal protein loading. Blots are representative of two independent experiments.

doi:10.1371/journal.pone.0044363.g006
tion point in promoting receptor internalization to regulate EGFR surface expression.

Our data indicating that Cdc42 stimulates direct ACK1 phosphorylation of cortactin is in agreement with the localization of these proteins at clathrin coated pits during early endocytic stages, supporting cortactin as an ACK1 substrate during receptor internalization [17,29,63]. We show that ACK1 phosphorylates the canonical cortactin tyrosine residues 421/466/482 that are also targeted by Src [64], Abl/Arg [36], Fer [65], c-Met [66], Fyn [67] and HER2 [68]. Src activation triggered by EGFR contributes to receptor endocytosis and is responsible for simulating ACK1 activity [69,70]. Src phosphorylation of cortactin is required for transferrin receptor CME [33,34], indicating that cortactin is also targeted by Src during endocytosis. In addition, Abl is activated during EGFR stimulation but plays a negative role in receptor downregulation [12].

The resultant aggregate activity of multiple kinases towards the same cortactin phosphorylation sites during EGFR endocytosis increases the level of complexity regarding regulation of endocytic events controlled by cortactin tyrosine phosphorylation. Nevertheless, our direct phosphorylation data with purified proteins combined with localization of ACK1 and cortactin with activated EGFR suggests that at least a subset of cortactin is likely phosphorylated by ACK1 during receptor internalization. The additional low level ACK1 phosphorylation present in cortactin lacking tyrosines 421/466/482 may be due to targeting of other tyrosine residues phosphorylated downstream of EGFR [71] or possible phosphorylation of serine/threonine sites due dual specificity of ACK1 [72]. Identification of these additional cortactin phosphorylation sites targeted by ACK1 remains to be determined.

Deletion mapping and point mutational studies indicate that the cortactin SH3 domain mediates binding to ACK1. Analysis of the ACK1 primary sequence by the STRING database (http://string.embnet.de) indicates that the ACK1 proline rich region contains a region between amino acids 735–743 (KPQVPRPRVP) that is a partial consensus with proline rich regions in other proteins that bind the cortactin SH3 domain [73]. We are currently working on identifying the precise cortactin binding site in ACK1 within the proline rich region. In addition to ACK1, the cortactin SH3 domain binds to proline rich sequences in CD2AP [39] and dynamin2 [29] during CME. The multiplex binding of the cortactin SH3 domain to proteins with different functions likely delineates specific roles during endocytic receptor internalization. Cortactin SH3 domain binding to CD2AP links Arp2/3 actin nucleation to EGFR during internalization through a CD2AP complex containing Chl and endophilin [39]. Endophilin contains an N-BAR (Bin–amphiphysin–Rvs) domain that inserts into the cytoplasmic plasma membrane face, inducing membrane curvature to initiate vesicle formation [74]. The interaction of cortactin with dynamin2 has been shown to regulate fission of clathrin coated vesicles through Src phosphorylation of cortactin and concurrent regulation of actin dynamics [28,32,34]. Cortactin binding to ACK1 likely occurs later in the internalization pathway, since ACK1 localizes at the base of exaggerated elongated tubular invaginations reminiscent of endosomes present in dynamin-null cells [17] as well as on early endosomes in other cell types [15]. The sequential binding of cortactin SH3 domain proteins is supported by a comprehensive analysis of CME using live cell imaging, where peak recruitment of dynamin2 occurred before ACK1 to the preassociated cortactin-containing F-actin network [75]. However, dynamin2 has recently been shown to also regulate late stages of endosomal budding [76], suggesting overlapping and redundant functions of cortactin SH3 domain binding proteins in receptor internalization and trafficking.

The findings that ACK1 and the cortactin SH3 domain are required for EGFR degradation point to a functional role for this complex in regulating EGFR surface expression. The loss of cortactin tyrosine phosphorylation during receptor downregulation (Figure 5A) suggests that ACK1-targeted cortactin tyrosine residues are dephosphorylated while ACK1 is still bound to cortactin. EGFR-containing late endosomes are targeted to MVBs, where dephosphorylation of tyrosine residues in the EGFR cytoplasmic domain by the ER-associated protein tyrosine phosphatase PTP1B triggers subsequent lysosomal targeting and degradation [77]. Fusion of late endosomes containing phosphorylated cortactin and ACK with MVBs would place cortactin in close proximity to PTP1B, which dephosphorylates cortactin [71]. Dephosphorylation of cortactin would be released from the vesicle surface and recycled with ACK1, or ACK1 may remain constitutively bound to and degraded with EGFR [21,23]. While dephosphorylation of cortactin would remove Nck1 docking sites required for the assembly of N-WASp/WIP Arp2/3 nucleation complexes, cortactin remains phosphorylated on the ERK1/2 phosphorylation sites important for maintaining the SH3 domain in an “open” conformation [52,60]. This would allow binding to N-WASp and presumably other SH3 domain binding proteins, providing for and maintaining Arp2/3-based actin polymerization during vesicle trafficking [78].

In summary, we have demonstrated that cortactin is an ACK1 substrate that potentially links the ACK1 EGFR internalization pathway to Arp2/3-based cortical actin regulation. It is likely that this pathway plays important roles in tumor progression, since elevated expression of ACK1 and cortactin due to gene amplification is found in several tumor types [79,80], and dysregulation of ACK1 and cortactin expression alters EGFR internalization dynamics [18,40]. How cortactin tyrosine phosphorylation is regulated by ACK1 and other cortactin kinases during internalization, as well as an improved understanding how the binding of cortactin SH3 domain-associated proteins are orchestrated are important questions for future consideration.

Supporting Information

**Figure S1** RhoA activation does not impact cortactin tyrosine phosphorylation. COS1 cells were cotransfected with FLAG-cortactin WT and Myc-tagged RhoA constructs as indicated. EV; empty vector. Cells were lysed 18 h after transfection, FLAG-cortactin immunoprecipitated and total cortactin tyrosine phosphorylation.

**Figure S2** Specificity of the Myc-ACK1-cortactin co-immunoprecipitation. 293T cells transiently transfected with Myc-ACK1 were lysed and immunoprecipitated with Protein A/G beads alone (Beads Only) or with Protein A/G beads bound to anti-cortactin antibody (Anti-CITTN). Immune complexes were resolved by SDS-PAGE and blotted for ACK1 and cortactin (Cort) as indicated. A non-specific binding product (NS) is shown as a control for equal loading. Blots are representative of two independent experiments.
Figure S3 Cortactin localizes with vesicles containing activated EGFR. 1433 cells were serum starved for 16 h and then left either untreated (No Tx) or stimulated with AlexaFlour-488 EGF (100 nanograms/milliliter, green) for 30 min before fixation. Cells were stained with anti-EGFR (red) and anti-cortactin (blue) antibodies. Confocal images of labeled EGFR in the apical (top) and ventral (bottom) cellular regions are shown. Scale bars, 20 micrometers. (TIF)

Figure S4 ACK1 is not a component of cortactin-containing invasive subcellular structures. (A) 584 cells cotransfected with activated Src kinase (527F) and Myc-ACK1 were plated on coverslips, fixed, and labeled with rhodamine phallolidin (Actin), anti-Myc (blue) and anti-cortactin (green) antibodies. Src-induced podosome rosettes (left panels) are identified as yellow circular aggregates; individual invadopodia (right panels) as subnuclear ventral puncta in the merged actin/cortactin images (white arrows). Arrowheads denote actin/cortactin containing lamellipodia. (B) 1433 or 364 cells were transfected with non-targeting siRNA (Ctrl) or siRNA targeting ACK (Si) and analyzed by Western blotting with anti-ACK1 and anti-actin antibodies. (C) 584 cells expressing activated Src were plated on FITC-gelatin covered coverslips (psuedocolored white) for 24 h, fixed and labeled with rhodamine phallolidin (Actin) and anti-cortactin (green) antibodies. Scale bar, 20 micrometers. (TIF)

Acknowledgments

The authors thank Joe Bolen and Jeng-Hong Her (DNAX Research Institute) for the ACK1 cDNA, Mike Webber (University of Virginia) for COS1 cells, Julie Head, Kimberly Irby and Kurt Christensen (University of Colorado, Denver) for technical assistance and J. Thomas Parsons and Yun-Rui Du (University of Virginia) for early advice. The contributions of Karen Martin and the West Virginia University Microscope Imaging Facility, Mary Babb Randolph Cancer Center are gratefully acknowledged.

Author Contributions

Conceived and designed the experiments: LCK SAW. Performed the experiments: LCK SAW. Analyzed the data: LCK SAW. Contributed reagents/materials/analysis tools: LCK SAW. Wrote the paper: LCK SAW.

References

1. Le Roy C, Wanra JL (2005) Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. Nat Rev Mol Cell Biol 6: 112–126.
2. Sorokin A, Goh LK (2005) Endocytosis and intracellular trafficking of ErbBs. Exp Cell Res 313: 681–696.
3. Jiang X, Huang F, Marnuy A, Sorokin A (2003) Grb2 regulates internalization of EGF receptors through clathrin-coated pits. Mol Cell Biol 14: 858–870.
4. Levkowitz G, Waterman H, Zanin E, Kam Z, Oved S, et al. (1998) c-If/Sl-i regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev 12: 3663–3674.
5. Goh LK, Huang F, Kim W, Gygi S, Sorokin A (2010) Multiple mechanisms collectively regulate cortactin-mediated endocytosis of the epidermal growth factor receptor. J Cell Biol 189: 871–883.
6. Sugimori S, Weolk T, Purci C, Maperos E, Tacchieri C, et al. (2005) Clathrin-independent endocytosis of ubiquitinated cargo. Proc Natl Acad Sci U S A 102: 2760–2765.
7. Yamazaki T, Zaal K, Hailey D, Presley J, Lippincott-Schwartz J, et al. (2006) Role of Grb2 in EGF-stimulated ERK-IR4 internalization: J Cell Sci 115: 1791–1802.
8. Orth JD, Krueger EW, Wellger SG, McNiven MA (2006) A novel endocytic mechanism of epidermal growth factor receptor sequestration and internalization. Cancer Res 66: 3603–3610.
9. Hynes NE, MacDonald G (2006) ErbB receptors and signaling pathways in invasion of human breast cancer cells. Breast Cancer Res 10: R36.
10. Kalyankrishna S, Grandis JR (2006) Epidermal growth factor receptor biology in lung cancer. J Mol Med 84: 739–749.
11. Lambert DJ, Grandis JR (2006) Biology of the epidermal growth factor receptor. Alcohol Clin Exp Res 314: 1292–1300.
12. Chen H, Li J, Wang X, Li F, Kong L, et al. (2007) Dysregulation of Ack1 inhibits down-regulation of the EGF receptor. Exp Cell Res 313: 202–210.
13. Liu Q, Lu CG, Cerione RA, Yang W (2002) The Cde42 target ACK2 interacts with sorting nexin 9 (SH3PX1) to regulate epidermal growth factor receptor degradation. J Biol Chem 277: 10134–10138.
14. Chan W, Tian R, Lee VF, Sit ST, Lim I, et al. (2009) Down-regulation of active ACK1 is mediated by association with the E1 ubiquitin ligase Nedd4-2. J Biol Chem 284: 8185–8194.
15. Chu BT, Lim SJ, Tham SC, Poh WJ, Ulrich A (2010) Somatic mutation in the ACK1 ubiquitin association domain enhances oncospecific signaling through EGFR regulation in renal cancer derived cells. Mol Oncol 4: 323–334.
16. Lin W, Wang J, Childress C, Sudol M, Carey DJ, et al. (2010) HECT E3 ubiquitin ligase Nedd4-1 ubiquitates ACK and regulates epidermal growth factor (EGF)-induced degradation of EGF receptor and ACK. Mol Cell Biol 30: 1541–1554.
17. Schaefer DA (2002) Coupling actin dynamics and membrane dynamics during endocytosis. Curr Opin Cell Biol 14: 76–81.
18. Qualmann B, Kessels MM, Kelly RB (2000) Molecular links between endocytosis and the actin cytoskeleton. J Cell Biol 150: F111–116.
19. Pollard TD (2007) Regulation of actin filament assembly by Arp2/3 complex and formins. Annu Rev Biophys Biomol Struct 36: 451–477.
20. Rottner K, Hansich J, Campellone KG (2010) WASH, WHAMM and JMY: regulation of Arp2/3 complex and beyond. Trends Cell Biol 20: 650–661.
21. Merrifield CJ, Perrias D, Zemiek B (2005) Coupling between clathrin-coated-pit invagination, cortactin recruitment, and membrane scission observed in live cells. J Cell Biol 169: 593–606.
22. Cao H, Orth JD, Chen J, Wellger SG, Heuser JE, et al. (2003) Cortactin is a component of clathrin-coated pits and participates in receptor-mediated endocytosis. Mol Cell Biol 23: 2162–2170.
23. Kaksonen M, Peng HB, Raunava H (2000) Association of cortactin with dynamic actin in lamellipodia and on endosomal vesicles. J Cell Sci 113 Pt 24: 4421–4426.
24. McNiven MA, Kim L, Krueger EW, Orth JD, Cao H, et al. (2000) Regulated interactions between dynamin and the actin-binding protein cortactin medulate cell shape. J Cell Biol 151: 167–198.
25. Zhu J, Zhou K, Hao J, Liu J, Smith N, et al. (2005) Regulation of cortactin/dynamin interaction by actin polymerization during the fission of clathrin-coated pits. J Cell Sci 118: 807–817.
26. Zhu J, Yu D, Zeng XC, Zhou K, Zhan X (2007) Receptor-mediated endocytosis involves tyrosine phosphorylation of cortactin. J Biol Chem 282: 16036–16044.
27. Cao H, Chen J, Krueger EW, McNiven MA (2010) SRC-mediated phosphorylation of dynamin and cortactin regulates the “constitutive” endocytosis of transferrin. Mol Cell Biol 30: 781–792.
28. Tehrani S, Tomasevic N, Weed S, Sakowicz R, Cooper JA (2007) Src phosphorylation of cortactin enhances actin assembly. Proc Natl Acad Sci U S A 104: 11933–11938.
29. Boyle SN, Michaud GA, Schweitzer B, Predki PF, Koleske AJ (2007) A critical role for cortactin phosphorylation by Abl-family kinases in PDGF-induced dorsal-wave formation. Curr Biol 17: 445–451.
30. Le Clainche C, Pauly BS, Zhang CX, Engqvist-Goldstein AE, Cunningham K, et al. (2007) A Hip1R-cortactin complex negatively regulates actin assembly associated with endocytosis. EMBO J 26: 1199–1210.
58. Artym VV, Zhang Y, Seillier-Moiseiwitsch F, Yamada KM, Mueller SC (2006) Sorting of ligand-activated epidemial growth factor receptor to lysosomes requires its actin-binding domain. J Biol Chem 279: 11562–11569.
59. Lynch DK, Vinata SC, Lyons RJ, Hughes WE, Lehrbach GM, et al. (2003) A Cortactin-CBD-associated protein (CD2AP) complex provides a novel link between epidermal growth factor receptor endocytosis and the actin cytoskeleton. J Biol Chem 278: 21805–21815.
60. Timpson P, Lynch DK, Schramek D, Walker F, Daly RJ (2005) Cortactin overexpression inhibits ligand-induced down-regulation of the epidemial growth factor receptor. Cancer Res 65: 3273–3280.
61. Sacks PG, Parmes SN, Gallick GE, Mansouri Z, Lichtner R, et al. (1988) Establishment and characterization of two new squamous cell carcinoma cell lines derived from tumors of the head and neck. Cancer Res 48: 2163–2166.
62. Singh B, Gogineni SK, Sacks PG, Shaha AR, Shah JP, et al. (2001) Molecular cytogenetic characterization of head and neck squamous cell carcinoma and refinement of 3q amplification. Cancer Res 61: 4506–4513.
63. Rothshild BH, Shin AH, Ammer AG, Kelley LC, Ibray KB, et al. (2006) Cortactin overexpression regulates actin-related protein 2/3 complex activity, motility, and invasion in carcinomas with chromosome 11q13 amplification. Cancer Res 66: 8017–8025.
64. Olson MF, Pasteris NG, Gorski JL, Hall A (1996) Faciogenital dysplasia protein (FGD1) and Vav, two related proteins required for normal embryonic development, are upstream regulators of Rho GTPases. Curr Biol 6: 1628–1633.
65. Weed SA, Karginov AV, Schafer DA, Weaver AM, Kinley AW, et al. (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.
66. Du Y, Weed SA, Xiong WC, Marshall TD, Parsons JT (1998) Identification of a novel cortactin SH3 domain-binding protein and its localization to growth cones of cultured neurons. Mol Biol Cell 18: 5383–5401.
67. Kelley LC, Ammer AG, Hayes KE, Martin KH, Machida K, et al. (2010) Oncogenic Src requires a wild-type counterpart to regulate invadopodia maturation. J Cell Sci 123: 3923–3932.
68. Kinley AW, Weed SA, Weaver AM, Karginov AV, Bissonette E, et al. (2003) Cortactin interacts with WIP in regulating Arp2/3 activation and membrane protrusion. Curr Biol 13: 384–393.
69. Sheffield P, Garrard S, Derevenska Z (1999) Overcoming expression and purification problems of RhoGDI using a family of "parallel" expression vectors. Protein Expr Purif 15: 54–59.
70. Head JA, Jiang D, Li M, Zorn LJ, Schaefer EM, et al. (2003) Cortactin tyrosine phosphorylation requires Rac1 activity and association with the cortical actin cytoskeleton. Mol Biol Cell 14: 3216–3229.
71. Wu H, Reynolds AB, Kanner SB, Vines RR, Parsons JT (1991) Identification and characterization of a novel cortactin-associotated pp60src substrate. Mol Cell Biol 11: 3123–3124.
72. Kelley LC, Hayes KE, Ammer AG, Martin KH, Weed SA (2010) Cortactin phosphorylated by EKR1/2 localizes to sites of dynamic actin regulation and is required for carcinoma lamellipodia persistence. PLoS One 5: e13847.
73. Manser E, Leung T, Salihuddin H, Tan L, Lim L (1993) A non-receptor tyrosine kinase at invadopodia: defining the stages of invadopodia formation and function. Cancer Res 63: 3034–3043.
74. Campbell DH, Sutherland RL, Daly RJ (1999) Signaling pathways and structural domains required for phosphorylation of EMM1/cortactin. Cancer Res 59: 5376–5385.
75. Martinez-Queso N, Ho HY, Kirschner MW, Ramesh N, Geha RS (2004) Erk/Src phosphorylation of cortactin acts as a switch on-switch off mechanism that controls its ability to activate N-WASP. Mol Cell Biol 24: 5269–5280.
76. Eden ER, White JJ, Futter CE (2009) Down-regulation of epidemial growth factor receptor signalling within multivesicular bodies. Biochem Soc Trans 37: 173–177.
77. Ohashi E, Tanabe K, Henni Y, Mesaki K, Kobayashi Y, et al. (2011) Receptor Sorting within Endosomal Trafficking Pathway Is Facilitated by Dynamic Actin Filaments. PLoS One 6: e19492.
78. Hussain NK, Jenu S, Glogauer M, Quinn GC, Wasiak S, et al. (2001) Endocytic protein intersectin regulates actin assembly via Cdc42 and N-WASP. Nat Cell Biol 3: 927–932.
79. Huang C, Liu J, Hauserdicol CC, Zhan X (1998) The role of tyrosine phosphorylation of cortactin in the locomotion of endothelial cells. J Biol Chem 273: 25376–25376.
80. Kapus A, Di Ciano C, Sun J, Zhan X, Kim L, et al. (2000) Cell volume-dependent phosphorylation of proteins of the cortical cytoskeleton and cell-cell contact sites. The role of Fyn and FER kinases. J Biol Chem 275: 32289–32296.
81. Crostella L, Liischer S, Williams K, Strouten G (2001) Haptocytos. Growth Factor scatter factor induces phosphorylation of cortactin in A431 cells in a Src kinase-independent manner. Oncogene 20: 3735–3745.
82. Huang J, Asawa T, Takato T, Sakai R (2003) Cooperative roles of Fyn and cortactin in cell migration of metastatic murine melanoma. J Biol Chem 278: 48367–48376.
83. Garcia-Castillo J, Pederseen K, Angelini PD, Bech-Serra JJ, Colone N, et al. (2009) HER2 carboxy-terminal fragments regulate cell migration and cortactin phosphorylation. J Biol Chem 284: 25302–25313.
84. Kasa A, Shima T, Okada M (2005) Role of Src family tyrosine kinases in the regulation of epidermal growth factor signaling in PCL12 cells. Genes Cells 10: 1173–1187.
85. Chan W, Sitt ST, Manser E (2013) The Cdc42-associated kinase ACK1 is not autoinhibited but requires Src for activation. Biochem J.
86. Stubbe M, Duhe N, Tremblay ML (2008) PTP1B regulates cortactin tyrosine phosphorylation by targeting Tyt446. J Biol Chem 282: 15740–15746.
87. Yokoyama N, Longheed J, Miller WT (2005) Phosphorylation of WASP by the Cdc42-associated kinase ACK1: dual hydroxynimino acid specificity in a tyrosine kinase. J Biol Chem 280: 42219–42226.
88. Weed SA, Parsons JT (2003) Cortactin: coupling membrane dynamics to cortical actin assembly. Oncogene 20: 6418–6434.
89. Kjærulf O, Brodin L, Jung A (2011) The structure and function of endophilin proteins. Cell Biochem Biophys 60: 137–154.
90. Taylor MJ, Perrais D, Merrifield CJ (2011) A high precision survey of the molecular dynamics of mammalian clathrin-mediated endocytosis. PLoS Biol 9: e1006004.
91. Schroeder B, Weller SG, Chen J, Billadeau D, McNiven MA (2010) A Dyn2-CIN85 complex mediates degradative traffic of the EGFR by regulation of late endosomal budding. EMBO J 29: 3039–3053.
92. Eden ER, White JJ, Tsapara A, Futter CE (2010) Membrane contacts between endosomes and ER provide sites for PTP1B-epidermal growth factor receptor interaction. Nat Cell Biol 12: 267–272.
93. Grassart A, Meas-Yedid V, Guisard J, Ollivier-Marin JC, Duarte-Varsat A, et al. (2010) Pak1 phosphorylation enhances cortatin-N-WASP interaction in clathrin-caveolin-independent endocytosis. Traffic.
94. van der Horst EH, Degenhardt YY, Strelow A, Slavin A, Chinn L, et al. (2005) Sorting of ligand-activated epidermal growth factor receptor within endosomes and ER provide sites for PTP1B-epidermal growth factor receptor interaction. Nat Cell Biol 12: 267–272.
95. Grassart A, Meas-Yedid V, Dufoir A, Ollivier-Marin JC, Duarte-Varsat A, et al. (2010) Pak1 phosphorylation enhances cortatin-N-WASP interaction in clathrin-caveolin-independent endocytosis. Traffic.
96. van der Horst EH, Degenhardt YY, Strelow A, Slavin A, Chinn L, et al. (2005) Sorting of ligand-activated epidermal growth factor receptor within endosomes and ER provide sites for PTP1B-epidermal growth factor receptor interaction. Nat Cell Biol 12: 267–272.
97. Grassart A, Meas-Yedid V, Dufoir A, Ollivier-Marin JC, Duarte-Varsat A, et al. (2010) Pak1 phosphorylation enhances cortatin-N-WASP interaction in clathrin-caveolin-independent endocytosis. Traffic.
98. van der Horst EH, Degenhardt YY, Strelow A, Slavin A, Chinn L, et al. (2005) Sorting of ligand-activated epidermal growth factor receptor within endosomes and ER provide sites for PTP1B-epidermal growth factor receptor interaction. Nat Cell Biol 12: 267–272.
99. Grassart A, Meas-Yedid V, Dufoir A, Ollivier-Marin JC, Duarte-Varsat A, et al. (2010) Pak1 phosphorylation enhances cortatin-N-WASP interaction in clathrin-caveolin-independent endocytosis. Traffic.