Arg interacts with cortactin to promote adhesion-dependent cell edge protrusion

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INTRODUCTION

Carefully controlled cell movement is essential for diverse biological events such as embryogenesis, wound healing, and proper brain development, whereas aberrant cell migration underlies numerous pathological states such as inflammatory diseases and cancer metastasis. Directed cell migration requires changes in cell shape powered by dynamic rearrangements of the actin cytoskeleton. Actin polymerization promotes protrusions at the cell edge (Mitchison and Cramer, 1996; Pollard and Borisy, 2003; Ponti et al., 2004), whereas actomyosin networks direct cellular contraction to provide traction force for cell body translocation (Jay et al., 1995; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Ridley et al., 2003; de Rooij et al., 2005; Gupton and Waterman-Storer, 2006). These rearrangements are guided locally by extracellular cues that bind cell surface receptors to activate signaling pathways that control the actin cytoskeletal machinery.

Abelson (Abl) family kinases, which include the vertebrate Abl/Abl1 and Abl-related gene (Arg)/Abl2 proteins, are critical mediators of cytoskeletal rearrangements in response to growth factor or adhesion receptor engagement (Plattner et al., 1999, 2003, 2004; Woodring et al., 2002, 2004; Hernandez et al., 2004; Miller et al., 2004; Sini et al., 2004; Moresco et al., 2005). Several studies indicate that Abl family kinases promote localized actin network assembly in response to cell–cell or cell–ECM adhesion. For example, Abl family kinases stimulate actin-based cell edge protrusions in fibroblasts (Woodring et al., 2002, 2004) and neurite branching in neurons (Woodring et al., 2002; Moresco et al., 2005) as they adhere and spread on ECM molecules. Abl family kinases also promote actin assembly during immune synapse formation between B and T lymphocytes (Huang et al., 2008) and strengthen F-actin networks that connect adherens junctions (Zandy et al., 2007). Abl family kinases can phosphorylate diverse cytoskeletal effector proteins including the Dok (downstream of the Tyr kinase) family adapters (Cong et al., 1999; Master et al., 2003; Woodring et al., 2004), Abl-interacting (Abl) family proteins (Dai and Pendergast, 1995; Shi et al., 1995; Biesova et al., 1997), Enabled/mammalian...

Abbreviations used in this paper: Abl, Abelson; ANOVA, analysis of variance; Arg, Abl-related gene; cortactin-P, phosphorylated cortactin; dko, double knock-out; FL, full length; KD, knockdown; KI, kinase inactive; NTA, N-terminal acidic; N-WASP, neural Wiskott-Aldrich syndrome protein; PSD, protected least significant difference; SH, Src homology; WT, wild type.

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Results

Arg and cortactin colocalize to adhesion-induced protrusive structures

We have previously reexpressed Arg-YFP at endogenous levels in arg<sup>-/-</sup> fibroblasts (arg<sup>-/-</sup> + Arg-YFP cells) to demonstrate that Arg localizes to and promotes dynamic cell edge protrusions during fibroblast adhesion and spreading on fibronectin (Miller et al., 2004; Peacock et al., 2007). In this study, we retransformed a functional cortactin-RFP fusion protein (Boyle et al., 2007) in arg<sup>-/-</sup> + Arg-YFP cells and observed the localization of both proteins in near simultaneous time-lapse imaging during fibroblast adhesion and spreading on glass coverslips coated with 10 µg/ml fibronectin. Arg-YFP and cortactin-RFP localize to protrusive lamellar structures at the cell periphery (Fig. 1, compare B with C [enlarged in E and F]; and Video 1). A portion of cortactin-RFP was also found associated with intracellular vesicular structures, which is consistent with a well-established role for cortactin in endocytosis and vesicle trafficking (Cao et al., 2003; Merrifield et al., 2005). Interestingly, both Arg-YFP and cortactin-RFP localize to the cell periphery (Fig. 1, E and F, arrowheads) just before the formation of a protrusion (Fig. 1, E and F, asterisks). We find that in 86% (12/14) of the cases in which we observed newly formed areas of Arg–cortactin colocalization (n = 4 cells), they were associated with subsequent protrusion. These colocalization experiments suggest that Arg and cortactin may interact to promote adhesion-dependent cell edge protrusions.

Cortactin knockdown (KD) abrogates adhesion-dependent cell edge protrusion

Wild-type (WT) fibroblasts exhibit frequent dynamic cell edge protrusions as they adhere and spread on fibronectin-coated surfaces. These protrusions occur much less frequently in arg<sup>-/-</sup> fibroblasts, a defect which can be repaired by Arg-YFP reexpression (see Fig. 5, B–D; Miller et al., 2004). Similarly, we used kymography (Hinz et al., 1999) to determine whether cortactin is required for adhesion-dependent cell edge protrusion. During spreading on fibronectin, WT cells or WT cells expressing an empty short hairpin RNA vector (WT + vector) exhibit frequent dynamic protrusions and retractions of the cell edge, yielding kymographs that resemble “rolling hills” (Fig. 2, B, C, I, and J). In contrast, cortactin KD cells, which retain only 20% of endogenous cortactin (Fig. S1 E), exhibit a significantly reduced number of protrusions and retractions (Fig. 2 D), yielding flat, “prairie-like” kymographs. This deficiency was restored via reexpression of a functional RNAi-resistant cortactin monomeric RFP (cortactin-RFP) fusion (Fig. 2 E). These data indicate that cortactin, like Arg, is required for dynamic cell edge protrusion during fibroblast adhesion on fibronectin.

The cortactin SH3 domain binds to one of several PXXP motifs in Arg

We used a bead affinity pull-down assay to characterize the interactions between Arg and cortactin. We incubated a constant concentration of cortactin-agarose beads with increasing Arg concentrations. After allowing binding to reach equilibrium, the amount of cortactin-bound Arg or Arg fragments was measured (Fig. 3 A). Arg binds to cortactin and was retained on the beads in a concentration-dependent manner, but only trace amounts of Arg were recovered on the control beads (Fig. 3 A).

To identify the domains and motifs that mediate Arg binding to cortactin, we next measured binding affinities of various Arg truncation mutants for cortactin (Fig. 3 and Fig. S3).
SH3 domain fusion protein binds Arg 557-C with a similar affinity as FL cortactin ($K_d = 0.44 \pm 0.14 \mu M$), but mutation of a key binding residue in the cortactin SH3 domain (W525A) eliminates binding of cortactin to Arg 557-C (Fig. 3 E and Fig. S3, E and F). Mutation of either all three PXXP motifs or just PXXP1 in Arg 557-C (Arg 557-C mut1) disrupts its binding of the cortactin SH3 domain (Fig. 3 G and Fig. S3, G and H). It is important to note that the PXXP1 motif in Arg (residues 567–576) is a tandem array of PXXP motifs (PXXPXXPXXP). Only the central pair of Pro in this motif is conserved in Abl and other Abl family kinases. Mutation of all four Pro in this set (e.g., AXXAXXAXXAXX) was required to disrupt the ability of Arg 557-C to bind the cortactin SH3 domain or to support adhesion-dependent protrusions (Fig. 3 G and Fig. S3, G and H; see Fig. 5 I). However, mutation of both PXXP2 (P622A and P625A) and PXXP3 (P664A and P667A; Arg 557-C mut23) leads to only a modest reduction in binding of Arg 557-C to the cortactin SH3 domain ($K_d = 1.25 \pm 0.25 \mu M$; Fig. 3 G and Fig. S3 I). Together, these data indicate that cortactin binds to the Arg PXXP1 motif via its SH3 domain.

The Arg N-terminal half containing its SH3, SH2, and kinase domains (ArgΔC) does not bind cortactin (Fig. 3 C and Fig. S3 B). The Arg C-terminal half (Arg 557-C) contains a series of three PXXP motifs located in the linker region between the kinase domain and cytoskeletal-binding domains that could serve as potential binding sites for cortactin’s C-terminal SH3 domain. Arg 557-C binds to cortactin with submicromolar affinity ($K_d = 0.85 \pm 0.18 \mu M$; Fig. 3, A–C). Although Arg 557-C binds tightly to cortactin, a slightly smaller Arg fragment (Arg 688-C) lacking the PXXP motifs does not bind cortactin (Fig. 3 C and Fig. S3 D). We could not obtain full-length (FL) Arg at sufficient concentrations in solution to achieve binding saturation, and, thus, we can only place a lower limit ($K_d \geq 1.1 \mu M$) on this interaction (Fig. 3 C and Fig. S3 A). The reduced affinity of Arg for cortactin relative to Arg 557-C suggests that the Arg N-terminal half may restrict binding of cortactin to the Arg C-terminal half. Collectively, these data suggest that Arg residues 557–687 mediate cortactin binding.

Cortactin contains a C-terminal SH3 domain that might interact with the PXXP motifs in Arg. Purified GST-cortactin SH3 domain fusion protein binds Arg 557-C with a similar affinity as FL cortactin ($K_d = 0.44 \pm 0.14 \mu M$), but mutation of a key binding residue in the cortactin SH3 domain (W525A) eliminates binding of cortactin to Arg 557-C (Fig. 3 E and Fig. S3, E and F). Mutation of either all three PXXP motifs or just PXXP1 in Arg 557-C (Arg 557-C mut1) disrupts its binding of the cortactin SH3 domain (Fig. 3 G and Fig. S3, G and H). It is important to note that the PXXP1 motif in Arg (residues 567–576) is a tandem array of PXXP motifs (PXXPXXPXXP). Only the central pair of Pro in this motif is conserved in Abl and other Abl family kinases. Mutation of all four Pro in this set (e.g., AXXAXXAXXAXX) was required to disrupt the ability of Arg 557-C to bind the cortactin SH3 domain or to support adhesion-dependent protrusions (Fig. 3 G and Fig. S3, G and H; see Fig. 5 I). However, mutation of both PXXP2 (P622A and P625A) and PXXP3 (P664A and P667A; Arg 557-C mut23) leads to only a modest reduction in binding of Arg 557-C to the cortactin SH3 domain ($K_d = 1.25 \pm 0.25 \mu M$; Fig. 3 G and Fig. S3 I). Together, these data indicate that cortactin binds to the Arg PXXP1 motif via its SH3 domain.

Figure 1. Arg-YFP and cortactin-RFP colocalize to membrane protrusions in response to adhesion to fibronectin. Individual frames from time-lapse videos of arg−/− cells expressing Arg-YFP and cortactin-RFP (Video 1). (A–F) Phase-contrast (A and D), YFP channel (B and E), and RFP channel (C and F) images are shown. (D–F) Enlargements of the regions boxed in A, showing colocalization of Arg-YFP and cortactin-RFP (indicated by arrowheads) followed by the formation of a cell edge protrusion (indicated by asterisks). The elapsed time is shown in minutes:seconds. Bars, 10 µm.
Figure 2. Cortactin is required for adhesion-dependent cell edge protrusion. (A) Domain structure of RFP-tagged cortactin mutants used in this study. Cortactin has an NTA domain required for Arp2/3 binding followed by 6.5 cortactin repeats (shaded). A Pro-rich region (white box) contains three Tyr (Y) that can be phosphorylated by Arg and is followed by an SH3 domain. Cortactin 3F contains Tyr to Phe (F) point mutations in the phosphorylatable Tyr (Y421F, Y466F, and Y482F). The C-terminal SH3 domain is deleted in cortactin ΔSH3 (aa 1–494). Cortactin W525A contains a mutation in a key Trp in the SH3 domain (W525A) that abrogates SH3 binding to ligands. (B–H) Representative frames from 10-min time-lapse videos of cells plated on 10 µg/ml fibronectin. For kymography analysis, a radial grid of eight lines was placed over the phase images (as shown in B), and kymographs were constructed for each of the indicated lines. WT (n = 24 cells; B), WT expressing empty pSuper (pS) RNAi vector (n = 23 cells; C), cortactin KD (n = 26 cells; D), cortactin 3F–RFP (n = 20 cells; F), cortactin ΔSH3–RFP (n = 17 cells; G), and cortactin WA–RFP (n = 23 cells; H) are shown. (I and J) Quantification of the number of protrusions (I) and retractions (J) per 10-min video. Mean ± SEM. ANOVA between all cell types: protrusions, P < 0.0001; retractions, P < 0.0001. Fisher’s protected least significant difference (PLSD) for all cells versus WT: *, P ≤ 0.0002. Bars, 10 µm.

Adhesion stimulates Arg-dependent cortactin phosphorylation and creates a binding site for the Arg SH2 domain

Arg phosphorylates cortactin on three sites (Tyr Y421, Y466, and Y482) after stimulation of fibroblasts with PDGF (Boyle et al., 2007). This observation led us to investigate whether Arg phosphorylates cortactin upon adhesion to fibronectin. Adhesion of serum-starved WT fibroblasts to fibronectin stimulates
Figure 3. Cortactin uses its SH3 domain to bind to the first PXXP region of Arg. (A) Representative Coomassie blue-stained SDS-PAGE gel showing binding of the Arg C-terminal half (Arg 557-C) to cortactin beads but not to ethanolamine-blocked control beads. Arg 557-C binding to cortactin increases as a function of concentration, (B, D, and F) Plots of concentration (x axis) versus the amount bound (y axis) were fitted to a binding curve. Arg 557-C binding to cortactin (K_d = 0.85 ± 0.18 µM; n = 4; B), Arg 557-C binding to the cortactin SH3 domain (K_d = 0.44 ± 0.14 µM; n = 3; D), and Arg 557-C PXXP mut23 binding to the cortactin SH3 domain (K_d = 1.25 ± 0.25 µM; n = 2; F) are shown. (C, E, and G) Affinities of Arg and cortactin binding. Each binding reaction was tested at least twice, with combinations showing positive binding tested at least three times. (C) Affinity of Arg/Arg mutants for cortactin. Arg is composed of an N-terminal SH2 and SH3 domain followed by a kinase domain. This fragment does not bind to cortactin. Arg 557-C containing three conserved PXXP motifs (indicated by vertical lines), two F-actin–binding sites and a microtubule (MT)-binding site binds cortactin, whereas a shorter fragment lacking the Pro-rich stretch (Arg 688-C) does not. (E) Affinity of cortactin/cortactin fragments for Arg 557-C. See Fig. 2 for a description of the cortactin domains. The cortactin SH3 domain is necessary and sufficient to bind Arg 557-C. (G) Affinity of Arg 557-C PXXP mutants for the cortactin SH3 domain. Mutation of PXXP1 in Arg 557-C abrogates binding to the cortactin SH3 domain.

Tyr phosphorylation of cortactin threefold compared with cells held in suspension (Fig. 4 A). Arg is required for this phosphorylation, as adhesion to fibronectin does not stimulate cortactin phosphorylation in arg^-/- cells (Fig. 4 B) or in WT cells treated with the Abl/Arg kinase inhibitor STI-571 (Fig. 4 C), even though they adhere with similar kinetics to fibronectin-coated
Figure 4. Adhesion-dependent cortactin phosphorylation by Arg creates an additional Arg-cortactin-binding site. (A–D) Arg is required for adhesion-dependent phosphorylation of cortactin. (A–C) WT (n = 6 experiments; A), arg−/− cells (n = 3 experiments; B), or WT cells treated with STI-571 (n = 3 experiments; C) were plated on 10 µg/ml fibronectin, and cortactin was immunoprecipitated and immunoblotted with anti-phospho-Tyr (PY) or anti-cortactin antibodies.
To Arg 557-C (K...tion is mediated by the Arg SH2 domain, which binds cortactin
K...ing curves showing binding of Arg and Arg fragments to Tyr-phosphorylated cortactin (cortactin-P). Each binding reaction was performed in triplicate. (I) Arg
K...nent half (Arg 557-C). Cortactin-P binds to Arg 557-C with an affinity
K...d = 0.37 ± 0.07 µM; Fig. 4 J). This interaction is mediated by the Arg SH2 domain, which binds cortactin with similar high affinity (Kd = 0.24 ± 0.07 µM; unpublished data). Most notably, cortactin-P exhibits a greater than fivefold enhanced binding affinity for FL Arg (Kd = 0.21 ± 0.05 µM) compared with cortactin (Kd > 1.1 µM; Figs. 3 C and 4 K). Together, these experiments indicate that adhesion to fibronectin stimulates Arg-dependent cortactin phosphorylation, which creates a high affinity binding surface for the Arg SH2 domain.

Disruption of SH3-mediated cortactin binding to Arg compromises adhesion-dependent cell edge protrusion

The cell edge protrusion defects of arg−/− or cortactin KD cells can be quantitatively restored via reexpression of Arg-YFP (Miller et al., 2004) or cortactin-RFP (Fig. 2 E), respectively. We used this complementation assay to explore whether Arg–cortactin interactions mediate adhesion-dependent cell edge protrusions in vivo.

Each mutant was retrovirally expressed at levels similar to WT control cells (See Fig. S1 and Fig. S2 for quantification and Fig. S2 and Fig. S4 for localization of Arg-YFP and cortactin-RFP fusion proteins; Miller et al., 2004; Peacock et al., 2007). Although they localize normally to the cell periphery (Fig. S1, A–D), cortactin mutants lacking the C-terminal SH3 domain (∆SH3) or containing an inactivating point mutation in the SH3 domain (WA) that abrogates SH3-mediated binding to Arg (Fig. 3 E) do not support adhesion-dependent cell edge protrusions (Fig. 2, G–J). These data show that cortactin requires its Arg-binding SH3 domain to support adhesion-dependent cell edge protrusion.

An Arg C-terminal half fragment (Arg 557-C–YFP) can restore adhesion-dependent cell edge dynamics to arg−/− cells to 70% of WT levels (Fig. 5 G; Miller et al., 2004). Interestingly, a C-terminal Arg fragment (Arg 688-C–YFP) lacking the three PXXP motifs did not rescue adhesion-dependent cell edge protrusion (Fig. 5 H), even though it undergoes normal adhesion-dependent localization to the cell periphery (Fig. S4, G and J; Wang et al., 2001). Similarly, an Arg 557-C–YFP mutant in which all Pro residues in the three PXXP motifs were mutated to Ala (Arg 557-C PXXP mut123-YFP) does not support adhesion-dependent cell edge protrusions (Fig. S5). Significantly, mutation of the first PXXP motif only (Arg 557-C PXXP mut1-YFP) was sufficient to reduce cell edge protrusions to levels observed in arg−/− + YFP cells (Fig. 5 I). The Arg 557-C mutant in which only PXXP1 was intact (Arg 557-C PXXP mut23-YFP) supported adhesion-dependent protrusions to similar levels to Arg 557-C–YFP (Fig. 5 J). Coupled with the binding experiments presented in Fig. 3, these data strongly suggest that Arg PXXP1 binding to the cortactin SH3 domain is required for adhesion-dependent cell edge dynamics.

Disruption of cortactin phosphorylation reduces cell edge dynamics

Having shown that Arg is required for cortactin phosphorylation, which creates a binding site for the Arg SH2 domain, we
Figure 5. A Pro-rich region in the Arg C terminus is required for cell edge dynamics. [A] Graphical depiction of Arg mutants used in this experiment. See Fig. 3 for a description of the domains. KI, Kl (K317M) point mutation in the active site. ArgmutSH2 contains a point mutation (R176K) in the SH2 domain that abrogates binding to phospho-Tyr–containing binding partners. Arg 557-C PXXP mut1 and Arg 557-C PXXP mut23 contain point mutations in PXXP
tested whether cortactin phosphorylation is required for adhesion-dependent cell edge protrusion. The cortactin 3F phosphorylation site mutant rescued cell edge dynamics to only ~30% of the rescue level observed with WT cortactin (Fig. 2, F, I, and J). The rare protrusions that did form in these cells did not extend as far (2.0 ± 0.3 µM) as protrusions observed in WT cells (2.5 ± 0.2 µM). Similarly, an Arg SH2 domain–binding defective point mutant (R176K) could partially restore protrusion to arg⁻/⁻ cells (Fig. 5, A, F, K, and L) but only to the intermediate protrusion levels similar to those observed with Arg 557-C–YFP. Together with the aforementioned binding experiments, these data demonstrate that cortactin phosphorylation and its reinforcement of Arg–cortactin binding interactions are required to support robust adhesion-dependent cell edge protrusion.

Abl can substitute for Arg kinase activity but not for a kinase-independent Arg scaffold function in supporting adhesion-dependent protrusions

Surprisingly, when introduced into arg⁻/⁻ cells, a kinase–inactive (KI) catalytic point mutant of Arg (Arg KI-YFP) fully restored the protrusion and retraction rate to WT levels (Fig. 5 E). This observation seemed inconsistent with the requirements for Arg in adhesion-dependent cortactin phosphorylation (Fig. 4, A–C) and for cortactin phosphorylation for cell edge protrusion. We used serum-free conditions in adhesion-dependent phosphorylation assays to isolate the contribution of adhesion receptors. However, in contrast to the serum-free conditions of the adhesion-dependent phosphorylation assays, the cell edge protrusion assays must be performed in complete serum-containing medium to observe robust protrusion activity. We reasoned that in this context, some other kinase (e.g., Abl) may phosphorylate cortactin to allow cell edge protrusion. In support of this hypothesis, we find that the Abl/Arg inhibitor STI-571, which blocks adhesion-dependent cortactin phosphorylation (Fig. 4 C), inhibits adhesion-dependent cell edge protrusion (Fig. 6, A, B, and D). We noted a similar low level of adhesion-dependent protrusions in abl⁻/⁻ arg⁻/⁻ double knockout (dko) mouse fibroblasts expressing YFP (Fig. 6, A, B, and E; Koleske et al., 1998). Expression of Arg-YFP in dko cells restored adhesion-dependent protrusions nearly to levels observed in WT cells, whereas Abl-YFP does not support adhesion-dependent protrusions in dko cells (Fig. 6, A, B, F, and H). Although Arg KI-YFP could support adhesion-dependent protrusions in arg⁻/⁻ cells (Fig. 5, E, K, and L), it does not restore adhesion-dependent protrusions to dko cells (Fig. 6, A, B, and G). These data indicate that Arg provides two functions in supporting adhesion-dependent cell edge protrusion: a kinase activity that phosphorylates cortactin and a kinase-independent cortactin–binding scaffold function. Abl can substitute its kinase activity for that of Arg, but it is insufficient to supply the scaffold function.

A cortactin-Arg fusion protein can rescue protrusion defects in both arg⁻/⁻ and cortactin KD cells

Mutations that disrupt Arg–cortactin interactions compromise the abilities of the respective proteins to support adhesion-dependent protrusions. We reasoned that covalent fusion of otherwise noninteracting Arg and cortactin fragments might restore the ability of these proteins to support protrusions. Like FL Arg, the Arg 688-C fragment is targeted to the cell periphery via its interactions with F-actin and microtubules. However, because it lacks the PXXP motifs, Arg 688-C does not bind cortactin (Fig. 3 C, and Fig. S3 D) and therefore cannot support adhesion-dependent cell edge protrusion (Fig. 5, A, H, K, and L; Miller et al., 2004). Similarly, cortactinΔSH3 neither binds Arg nor supports protrusions (Fig. 2, A, G, I, and J). We tested whether fusion of these inert moieties could restore at least partial function to either arg⁻/⁻ or cortactin KD cell types. A cortactinΔSH3–Arg 688-C–RFP fusion protein was expressed at levels similar to endogenous Arg (Fig. S2 F) and localizes to the cell periphery and intracellular vesicles similarly to cortactin (Fig. S2, G and H). Interestingly, the cortactinΔSH3–Arg 688-C–RFP fusion protein restored adhesion-dependent protrusions to both arg⁻/⁻ and cortactin KD cells, even though these levels were only 40% or 45% of the levels supported by WT Arg or cortactin, respectively (Fig. 7, A, B, D, F, and G). Importantly, mutations of Tyr 421, 466, and 482 in the cortactin portion of the fusion protein (cortactin 3FΔSH3–Arg 688-C) abrogate its ability to rescue protrusions in either cell type (Fig. 7, A, C, and E–G). These data indicate that interactions between Arg and cortactin are necessary and at least partly sufficient to support protrusions in this context. These findings also further reinforce a model in which Arg acts as a scaffold to mediate downstream cortactin phosphorylation and function in the protrusion process.

The cortactin- and Arg-binding protein Nck1 is critical for cell edge protrusions

Cortactin phosphorylation enhances Arp2/3 complex–mediated actin nucleation in a purified recombinate system by potentiating interactions between cortactin and the Nck1 adapter protein (Tehrani et al., 2007). Interestingly, we also identified Nck1 in a high throughput screen for cortactin-P–binding proteins (unpublished data). We tested whether Nck1 is required for adhesion-dependent protrusions. Nck1 KD cells, in which >80% KD of Nck1 was achieved (Fig. 8 G), exhibited significantly less adhesion-dependent protrusive activity than WT cells (Fig. 8, B, D, and E). Interestingly, protrusion levels in Nck1 KD cells are lower than those observed in arg⁻/⁻ and cortactin KD cells.
Discussion

Abl family kinases act downstream of adhesion receptors to promote actin-based protrusions in a variety of contexts, but the molecular mechanisms by which they interface with the actin polymerization machinery are largely unclear. We present evidence...
that Arg interacts functionally with the Arp2/3 complex activator cortactin to promote adhesion-dependent cell edge protrusions. The cortactin SH3 domain binds to a PXXP motif in the Arg C terminus, and both features are required for adhesion-dependent cell edge protrusion formation. Adhesion stimulates Arg to phosphorylate cortactin, creating a novel high affinity binding site for the Arg SH2 domain and allowing Nck1 recruitment. Disruption of cortactin phosphorylation or Arg binding to phosphocortactin significantly impairs adhesion-dependent cell edge protrusion. Our data indicate that Arg acts as both a cortactin-binding scaffold and a cortactin kinase in the protrusion process. Under appropriate conditions, Abl can substitute for Arg to phosphorylate cortactin, but it cannot subserve the Arg scaffold function. Together, these data indicate that Arg and cortactin interact via both binding and catalytic events to promote cell edge protrusion during fibroblast adhesion and spreading.

**Abl/Arg-cortactin interactions mediate actin assembly in diverse biological contexts**

We demonstrate that Arg–cortactin interactions promote adhesion-dependent cell edge protrusion. Abl family kinases appear to interact with cortactin to promote actin assembly in a growing number of cellular contexts. For example, we showed previously that Abl- or Arg-mediated cortactin phosphorylation was required for PDGF-induced F-actin–rich circular dorsal ruffles (Boyle et al., 2007). Huang et al. (2008) have shown that Abl interacts with the hematopoietic cortactin homologue HS1 to regulate actin polymerization and lamellipodial spreading at the T cell immunological synapse. Both Abl family kinases and cortactin localize to epithelial adherens junctions where they may similarly interact to promote actin assembly critical to their stability (Helwani et al., 2004; Zandy et al., 2007; Zandy and

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**Figure 7.** A cortactin/Arg fusion protein lacking the SH3–PXXP interaction regions partially rescues adhesion-dependent cell edge protrusion. (A) Graphical depiction of a cortactin∆SH3–Arg 688-C–RFP fusion protein (Fusion) or the same with Tyr 421, 466, and 482 in cortactin mutated to Phe (Fusion 3F). MT, microtubule. (B–E) Representative images of 10-min time-lapse videos of cells spreading on glass coverslips covered with fibronectin. The three rightmost panes depict representative kymographs. Kymographs were constructed as in Fig. 2. arg−/− cells expressing the fusion protein (B) and the nonphosphorylatable fusion protein (Fusion 3F; C) and cortactin KD cells expressing the fusion protein (D), or the fusion 3F protein (E) are shown. (F and G) Quantitation of protrusions of either arg−/− cells (F) or cortactin KD cells (G) expressing the indicated constructs. Mean ± SEM; *, P < 0.05. Bars, 10 μm.
Arg functions primarily as a scaffold to promote protrusion formation

Despite our finding that Arg is required for adhesion-dependent cortactin phosphorylation, several observations suggest that Arg functions primarily in a scaffolding role to support cell edge protrusion. Indeed, we find that a KI Arg point mutant restores normal adhesion-dependent cell edge dynamics to 

\[\text{arg}^{-}\] cells. In fact, we find that the Arg C-terminal half, which lacks the SH3, SH2, and kinase domains, supports adhesion-dependent cell edge protrusions, albeit with reduced potency relative to the FL Arg.

Pendergast, 2008). Finally, Abl family kinases and cortactin both promote cancer cell invasiveness (Kain et al., 2003; Bowden et al., 2006; Hill et al., 2006; Rothschild et al., 2006; Srinivasan and Plattner, 2006; Clark et al., 2007; Clark and Weaver, 2008; Srinivasan et al., 2008). In this context, Abl and Arg may potentiate cortactin’s known ability to promote formation of invadopodia, cellular protrusions that invasive tumor cells use to degrade ECM, and basement membrane surrounding the tumor (Clark et al., 2007; Ayala et al., 2008; Clark and Weaver, 2008).

Figure 8. Nck1 is required for cell edge protrusion. (A) Graphical depiction of the Nck1 adapter protein. Nck1 consists of three SH3 domains followed by a single SH2 domain. (B and C) Representative frames from the 10-min time-lapse videos of cells plated on 10 µg/ml fibronectin-coated coverslips. The three rightmost panels are representative kymographs constructed as in Fig. 2. Nck1 KD cells \(n=29\) cells; B) and Nck1 KD cells expressing Nck-GFP \(n=19\) cells; C) are shown. (D and E) Quantification of the number of protrusions (D) and retractions (E) per 10-min video. Mean ± SEM. ANOVA between all cell types: protrusions, \(P<0.0001\); retractions, \(P<0.0001\). Fisher’s PLSD for all cells versus WT: *, \(P \leq 0.001\). (F) Localization of Nck1-GFP and cortactin in Nck1 KD cells. Nck1 KD cells were infected with Nck1-GFP (a and f) and endogenous cortactin was visualized by immunostaining (b and g). Cells were plated on fibronectin-coated coverslips and fixed 45 min after plating. F-actin was visualized with Alexa Fluor 350 phalloidin (c and h). The merged images of Nck1-GFP and cortactin images (d and i) are shown. The merged images of Nck1, cortactin, and actin (e and j) are also shown. F–j are enlargements of the areas indicated in a. (G) Immunoblot of WT and Nck1 KD cell lysates for Nck1. The HSP-70 panel represents a loading control. Bars, 10 µm.
In addition to its cortactin-binding PXXP1 motif, the Arg 557-C fragment contains both F-actin and microtubule-binding domains that mediate its proper localization to the cell periphery (Wang et al., 2001; Miller et al., 2004). Thus, the Arg C-terminal half serves as both a physical and functional bridge between the F-actin and microtubule cytoskeletons and the actin polymerization machinery.

**Does cortactin act as a scaffold for the assembly of actin regulatory complexes?**

The cortactin N-terminal half contains the NTA and cortactin repeats that mediate interactions with the Arp2/3 complex and F-actin, respectively (Weaver et al., 2001; for review see Weed and Parsons, 2001). These cortactin features are sufficient to weakly activate the Arp2/3 complex and to stabilize the resulting F-actin branch points (Urano et al., 2001; Weaver et al., 2001). Bryce et al. (2005) have shown that cortactin enhances lamellipodial persistence in human fibrosarcoma cells, and this function requires just the NTA and cortactin repeats. Together, these findings strongly suggest that cortactin’s role in F-actin branch formation/stabilization contributes to overall lamellipodial persistence.

The cortactin C-terminal half contains additional domains/motifs (e.g., phospho-Tyr residues, a Pro-rich region, and an SH3 domain) that can potentially mediate interactions with several cytoskeletal effectors, including the Arp2/3 activator N-WASp and the Wiskott-Aldrich syndrome protein–interacting protein (Mizutani et al., 2002; Kinley et al., 2003; for reviews see Weed and Parsons, 2001; Weaver et al., 2003; Daly, 2004; Cosen-Binker and Kapus, 2006). In this study, we demonstrate that the cortactin SH3 domain binds Arg. It is unclear how cortactin can use its SH3 domain to interact with both Arg and N-WASp or other proteins simultaneously. One possibility is that initial cortactin SH3 binding to Arg is remodeled to allow subsequent cortactin SH3 domain binding to N-WASp. Alternatively, Arg and N-WASp could bind simultaneously to SH3 domains on different cortactin molecules within the same Arp2/3 regulatory complex. A role for cortactin as an actin regulatory scaffold may explain the ability of the C-terminal cortactin fragment lacking the NTA and cortactin repeats to enhance migration of mammary epithelial cells (Kowalski et al., 2005).

**Cortactin phosphorylation reinforces Arg-cortactin interactions and may promote recruitment of additional cytoskeletal regulators**

We also show that the Arg SH2 domain can bind cortactin-P. This binding interaction significantly increases the overall affinity of Arg for cortactin by at least fivefold. Mutations that disrupt cortactin phosphorylation or Arg SH2 domain binding reduce protrusions to levels intermediate between WT and arg^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^\  

**Materials and methods**

**Molecular cloning and purification of recombinant protein**

Murine Arg and Arg mutant cDNAs were cloned into pFastBac expression vectors (QIAGEN), expressed, and purified as described previously (Wang et al., 2001). Murine cortactin and cortactin mutants were expressed and purified as described previously (Wang et al., 2001; Miller et al., 2004). Arg KO/YFP and Arg 557-C–YFP have been previously described (Miller et al., 2004). Arg 688-C–YFP was amplified by PCR from murine Arg using the amino acid numbering system for type IV myristoylated Arg and cloned into N1-EYFP (Clontech Laboratories, Inc.). The tagged sequence was then subcloned into the retinoblastoma cell line (QIAGEN), expressed, and purified as described previously (Miller et al., 2004; Boyle et al., 2007). Arg KI-YFP and Arg 557-C–YFP were maintained as previously described by (Miller et al., 2004). Arg 557-C–YFP was expressed and purified as described previously by (Boyle et al., 2007).

**Cell culture and retroviral expression**

WT, arg^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^\  

**Model**

We propose a model (Fig. 9) for how Arg interacts with cortactin to promote adhesion-dependent cell edge protrusion. (1) Arg localizes to the cell periphery where it uses its PXXP1 motif to bind the cortactin SH3 domain. (2) Adhesion stimulates Arg kinase activity. Arg phosphorylates cortactin, creating an additional high affinity binding site for Arg and possibly other proteins. In the absence of Arg, AbI is also capable of mediating cortactin phosphorylation in response to soluble cues. (3a) Phosphorylation-dependent remodeling of the Arg–cortactin complex allows the SH3 domain of cortactin to interact with N-WASp, stimulating actin polymerization and leading to cell edge protrusion. (3b) As an alternative or in addition, cortactin phosphorylation recruits other SH2 domain–containing proteins such as Nck1. Nck1’s three SH3 domains bind and activate N-WASp to promote actin polymerization (Rohatgi et al., 2001; Rivera et al., 2004), leading to cell edge protrusions.
and P667A (PXXP3). The cortactin-LSH3–Arg 688-C–RFP fusion protein was constructed by inserting PCR-amplified cortactinLSH3 sequence (aa 1–494) followed by a 5-aa linker (GSGGG) upstream of Arg 688-C into the monomeric RFP–N1 vector. This tagged fusion was subcloned into the PK1 expression vector. Expression levels of retrovirally expressed Arg or Arg mutants or the cortactinLSH3–Arg 688-C–RFP fusions were determined by semiquantitative immunoblotting of 1.5 µg/ml puromycin–selected cells, and the signal was compared with standardized concentrations of either purified Arg or purified YFP.

RNAi-mediated cortactin KD and rescue
Cortactin KD cells and rescue constructs for FL cortactin and cortactin 3F were previously described (Boyle et al., 2007). Cortactin WA contains a W525A mutation. All mutants were subcloned into the PK1 retroviral expression system. Expression levels of retrovirally expressed Arg or Arg mutants or the cortactinLSH3–Arg 688-C–RFP fusions were determined by semiquantitative immunoblotting of 1.5 µg/ml puromycin–selected cells, and the signal was compared with standardized concentrations of either purified Arg or purified YFP.

Immunofluorescence microscopy
arg+/− + Arg–YFP cells and cortactin KD cells expressing Arg/Arg mutant–YFP fusions or cortactin/cortactin mutant–RFP fusions were plated on glass coverslips coated with 10 µg/ml fibronectin (Invitrogen), fixed, stained, and imaged as previously described (Miller et al., 2004).

Time-lapse microscopy and kymography
For colocalization experiments, arg+/− + Arg–YFP cells were selected in 1.5 µg/ml puromycin and subsequently infected with RFP-tagged cortactin. Cells were adapted to growth in microscopy media (DME + 10% FBS and 10 mM Heps; Invitrogen) for ~16 h and imaged between 20 min and 2 h after plating using a microscope (TE2000-S; Nikon) driven by Openlab software (PerkinElmer). Cells were maintained at 37°C during imaging with an in-line flow heater and a heated chamber (Warner Instruments). 40× NA 1.0 objective (Nikon) phase-contrast and YFP videos were 10-min long with frames taken every 10 s with a cooled mono 12-bit camera (Retiga; QImaging). For colocalization experiments, exposures of the cells in the phase, YFP, and RFP channels were taken sequentially every 30 s. Kymography was performed as described previously (Miller et al., 2004) using ImageJ software (National Institutes of Health). Images and kymographs were cropped in Photoshop (Adobe) and were adjusted using the auto-level function. Statistical analysis of the protrusion data was performed by using analysis of variance (ANOVA) using StatView software (SAS Institute).

Adhesion assays and immunoprecipitation
WT and arg−/− cells were serum-starved in DME containing 0.1% FBS for at least 1 h and kept in suspension for 15 min. An equal volume of cells was harvested by centrifugation (for the 0 time point) or plated on tissue culture dishes coated with 10 µg/ml fibronectin. Cells were harvested by scraping at 20, 30, or 40 min after plating and lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 2 mM NaF, 1 mM Na3VO4, and protease inhibitors). Total protein concentration was determined using a biocinchonic acid protein assay kit (Thermo Fisher Scientific), and an equal amount of total protein per time point was subjected to immunoprecipitation with 2 µg anticalcineurin antibody (4F11; Millipore) and protein A/G beads (EMD). The immunoprecipitate was immunoblotted as described previously (Boyle et al., 2007). Phospho-Tyr and total cortactin levels were quantitated using ImageJ software (National Institutes of Health). Cells were maintained at 37°C during imaging with an in-line flow heater and a heated chamber (Warner Instruments). 40× NA 1.0 objective (Nikon) phase-contrast and YFP videos were 10-min long with frames taken every 10 s with a cooled mono 12-bit camera (Retiga; QImaging). For colocalization experiments, exposures of the cells in the phase, YFP, and RFP channels were taken sequentially every 30 s. Kymography was performed as described previously (Miller et al., 2004) using ImageJ software (National Institutes of Health). Images and kymographs were cropped in Photoshop (Adobe) and were adjusted using the auto-level function. Statistical analysis of the protrusion data was performed by using analysis of variance (ANOVA) using StatView software (SAS Institute).

Measurements of cortactin–Arg binding
Recombinant cortactin protein was covalently coupled to Affigel 15 resin (at a concentration of 1 mg/ml of gel bed [16.3 µM]; Bio-Rad Laboratories) according to the manufacturer’s protocol. The cortactin beads were washed twice with ice-cold binding buffer (50 mM Heps, pH 7.25, 125 mM...
NaCl, 0.01% NP-40, and 5% glycerol) and stored as a 50% slurry. Arg protein was diluted 1:3 in a serial fashion; 20 µl was saved as input sample, and 490 µl of each dilution was added to Eppendorf tubes containing 50 µl of cortactin bead slurry or 50 µl of blank bead slurry blocked with an excess of ethanolamine. The reaction was incubated at 4°C while rotating for 2 h. The supernatant was removed, and the beads were rapidly resuspended with 1 ml of ice-cold binding buffer. Bound material was recovered with 4x lysis sample buffer, analyzed on 8% SDSPAGE gels, stained with Coomassie blue R-250, and scanned using a densitometer (ImageQuant 1; Bio-Rad Laboratories). Intensity levels were adjusted using the auto-adjust feature in Quantity One software (Bio-Rad Laboratories). Integrated density measurements were made of the band as well as an empty area of the cell immediately above the band. This background measurement was subtracted from the band density measurement. Binding curves were generated using GraphPad Prism software (Bio-Rad Laboratories) using the equation

\[
Y = \frac{B_{\text{max}} \times X}{X + K_D + NS \times X}
\]

where Y equals the specific binding signal, X equals the concentration of ligand added to the cortactin beads, and NS equals the slope of the least-squares linear regression line for the specific binding as measured with the ethanolamine beads. For representative gels, the Photoshop auto-level function was used, and the gels were cropped to display the bands of interaction for each gel.

Recombinant cortactin-P was generated by coexpressing His-tagged cortactin with untagged Arg using baculoviral vectors in insect cells. Cell lysates were incubated with nickel beads, and three high salt washes (0.5 M, 1.25 M, 0.5 M KCl, respectively) were used to dislodge cortactin-bound Arg. Cortactin-P was eluted from the nickel column using 200 mM imidazole, and its phosphorylation status was verified via Western blotting with a cocktail of anti-phospho-Tyr antibodies. Recombinant SH2 domain binding was measured on cortactin affinity-fixed to nitrocellulose membranes as described previously (Nollau and Mayer, 2001; Machida et al., 2004). In brief, 0.4 µg in 1 µl dots of cortactin-P, cortactin 3F, pervanadate-treated cell lysate rich in phospho-Tyr-containing proteins, or phosphatase-treated cell lysate was spotted in duplicate on a nitrocellulose membrane and allowed to dry overnight at room temperature in a 6-well culture dish. Membranes were soaked in transfer buffer (20% MeOH, 12.5 mM Tris-HCl, pH 8.0, and 100 mM Gly) for 30 min, rinsed twice with TBST [150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 0.05% Tween 20], and blocked at room temperature for 1 h in TBST with 10% nonfat milk, 1 mM Na2VO4, and 1 mM EDTA. Purified recombinant GST-SH2 domains of Arg, Abl, and Grb2 conjugated to glutathione–horseradish peroxidase were added at a concentration of 1 µg/ml to separate chambers of the 6-well culture dish and allowed to incubate at room temperature for 1 h with constant orbital rotation. The membranes were then washed three times with TBST for 5 min each, dried, incubated with ECL, and exposed to film.

Online supplemental material

Fig. S1 shows the expression levels and localization of cortactin or cortactin mutants in cortactin KD cells. Fig. S2 shows the expression levels of Arg and Arg mutants in arg−/− cells. Fig. S3 shows that cortactin uses its SH3 domain to bind to the first PXPF region of Arg. Fig. S4 shows that Arg mutants colocalize with F-actin at the cell periphery. Fig. S5 shows that arg+/− cells expressing Arg 557C PXPF mut123 have reduced protrusion dynamics. Video 1 shows that Arg-YFP and cortactin-RFP colocalize to membrane protrusions in response to adhesion to fibronectin. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200809085/DC1.

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