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# A Crucial Role in Cell Spreading for the Interaction of Abl P<sub>xx</sub>P Motifs with Crk and Nck Adaptors

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## Essential role of c-Abl PxxP motifs and its interactions with Crk and Nck adaptors in cell spreading

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### Summary

The dynamic reorganization of actin structures helps to mediate the interaction of cells with their environment, and the c-Abl non-receptor tyrosine kinase can modulate actin rearrangement during cell attachment. Here we report that the c-Abl PxxP motifs, which bind Src Homology 3 (SH3) domains, are indispensable for the coordinated regulation of filopodium and focal adhesion formation and cell spreading dynamics during attachment. Candidate Abl PxxP motif binding partners were identified by screening a comprehensive SH3 domain phage display library. A combination of protein overexpression, silencing, pharmacological manipulation and mutational analysis demonstrated that the PxxP motifs of c-Abl exert their effects on actin organization by two distinct mechanisms, involving the inhibition of Crk signaling and the engagement of Nck. These results uncover a previously unappreciated role for c-Abl PxxP motifs in the regulation of cell spreading.

### Keywords

Abl PxxP motifs; Crk; Nck; filopodium and lamellipodium; cell spreading

### Introduction

The ability of cells to adhere and migrate in a temporally and spatially coordinated manner is critical for tissue morphogenesis, maintenance and repair. Regulated cell migration is driven by localized actin polymerization and the turnover of focal adhesions (Ridley et al., 2003). Local actin polymerization controls the formation of two distinctly organized F-actin structures, namely filopodia and lamellipodia. These are outward extensions of the membrane consisting of long and tightly bundled F-actin in the case of filopodia or a broad, highly branched F-actin meshwork in the case of lamellipodia (Mejillano et al., 2004; Ridley et al., 2003). Cells can sense extracellular cues and initiate locomotion through these protruding structures (Albrecht-Buehler, 1976; Izzard and Lochner, 1980). Productive cell migration requires the anchorage of newly formed membrane protrusions to the substratum through

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**Supplemental Online Material** The online supplemental material includes five display figures and three videos. The three videos consist of time-lapse DIC microscopy images of cells during early attachment. Video 1 shows Abl DKO cells and Abl DKO cells re-expressing wt c-Abl. Video 2 shows NIH3T3 cells overexpressing c-Abl, CrkII, or Nck2. Video 3 shows NIH3T3 cells in which Crk family adaptors have been knocked down.

incipient adhesions at the cell's leading edge and the disassembly of mature focal adhesions at the trailing edge (Ridley et al., 2003).

De novo nucleation and elongation of actin filaments by the Arp2/3 complex, Formins, and Spire promote the assembly of the actin meshwork (Baum and Kunda, 2005). It has been proposed that the Ena/Vasp family of proteins favors elongation and bundling of actin filaments resulting in formation of filopodia (Barzik et al., 2005; Bear et al., 2002; Svitkina et al., 2003). On the other hand, capping proteins are believed to limit the elongation of actin filaments and to promote branching, therefore, favoring the formation of lamellipodia (Mejillano et al., 2004). An important layer of regulation is provided by the Rho family of GTPases, which act as signaling nodes that integrate inputs from a variety of stimuli. They modulate the activity of proteins from the WASp/Scar family, which are actin nucleation promoting factors that regulate the Arp2/3 complex (Ridley, 2006).

Dynamic extension of filopodia and lamellipodia is observed during the attachment of cells to the substratum (Albrecht-Buehler, 1976) and c-Abl has been implicated in the formation of filopodia (Woodring et al., 2002). c-Abl and its close relative Arg, the two members of the Abl family of non-receptor tyrosine kinases, have partially overlapping functions in vivo (Kain and Klemke, 2001; Koleske et al., 1998; Zipfel et al., 2004). Structurally, Abl family proteins are comprised of a myristoylation signal, an SH3 domain (PxxP motif binding domain), an SH2 domain (phosphotyrosine binding domain), a tyrosine kinase catalytic domain, a cluster of PxxP motifs, and G- and F-actin binding domains (supplemental material Fig. S1A, (Pendergast, 2002)).

In the absence of activating signals, the kinase activity of c-Abl is suppressed by intramolecular interactions and interactions with other cellular proteins and inositol phospholipids (Hantschel and Superti-Furga, 2004; Pendergast, 2002; Woodring et al., 2003). During attachment on extracellular matrix components such as fibronectin, the suppression of c-Abl kinase activity is transiently relieved. It has been proposed that dissociation of c-Abl from F-actin leads to an increase in its catalytic activity during attachment (Woodring et al., 2001; Woodring et al., 2002). Activation of c-Abl kinase activity, which promotes filopodium formation, results in the phosphorylation of critical substrates including Dok1 (Woodring et al., 2002; Woodring et al., 2004).

A unique feature of the Abl family of non-receptor tyrosine kinases is the presence of a cluster of PxxP motifs capable of interacting with SH3 domain-bearing actin regulatory proteins such as those of the Abi, Crk, and Nck families (Dai and Pendergast, 1995; de Jong et al., 1995; Feller et al., 1994; Ren et al., 1994; Rivero-Lezcano et al., 1995; Smith et al., 1999). Virtually nothing is known, however, about the functional role of the c-Abl PxxP motifs and their interactions in biological activities such as the regulation of cytoskeletal rearrangement.

Here, we report that the cluster of PxxP motifs is indispensable for c-Abl to modulate filopodium formation, the dynamics of cell spreading, and focal adhesion formation during cell attachment. For these activities, Crk and Nck family adaptors are the critical effectors of the c-Abl PxxP motifs. Thus the binding of Crk and Nck family adaptors to its PxxP motifs enables c-Abl to fine-tune actin-based membrane dynamics as cells adapt to their surrounding environment.

## Results

### Role of c-Abl PxxP motifs in regulation of filopodium formation during attachment

The PxxP motifs of c-Abl have been shown to interact with a number of SH3-domain-containing proteins (Dai and Pendergast, 1995; de Jong et al., 1995; Feller et al., 1994; Lin et

al., 2001; Mitsushima et al., 2006; Ren et al., 1994; Shi et al., 1995; Smith et al., 1999; Wang et al., 1997). However, the physiological significance of such interactions remains largely unknown. To investigate the roles of the Abl PxxP motifs in transducing signals, we expressed various forms of Abl carrying mutations in the cluster of PxxP motifs (supplemental material Fig. S1A) in mouse embryonic fibroblasts lacking Abl and Arg (Abl DKO cells). To determine the expression levels of various Abl mutants in Abl DKO cells, western blotting was performed. After drug-selection and passing the cells for a month, the expression of wild-type (wt) c-Abl was lower than that of an Abl variant with mutations in all four PxxP motifs (1234) (Fig. 1A). Because high expression of wt c-Abl has been shown to inhibit cell growth and survival, cells expressing relatively low amounts of c-Abl are selected over time (Goga et al., 1993). Thus the relatively high level of expression of the 1234 mutant suggests it lacks some function necessary for growth inhibition.

c-Abl has been shown to increase filopodium formation during attachment on fibronectin-coated surfaces ((Woodring et al., 2002), Movie 1). Using Abl DKO cells expressing Abl variants, we first compared filopodium formation during attachment. Cells expressing the 1234 mutant had on average fewer filopodia compared to cells re-expressing wt c-Abl ( $2.54 \pm 0.21$  vs.  $8.80 \pm 0.47$  per cell, respectively) (Fig. 1B and C). The SH2 domain of c-Abl has been also shown to play an important role in filopodium formation (Woodring et al., 2004). We found that the decrease in filopodium formation seen with the 1234 mutant was more severe than that seen in cells expressing an Abl SH2 domain mutant deficient in phosphotyrosine binding (SH2\*) (Fig. 1B and C). We also consistently observed a further decrease in filopodium formation when the SH2\* mutation was combined with 1234, to a level indistinguishable from Abl DKO cells or those expressing a kinase-defective form of Abl (KD). These data indicate that the ability of c-Abl to induce filopodia depends strongly on its PxxP motifs. Furthermore, the cluster of PxxP motifs and the SH2 domain are likely to promote filopodium formation by different mechanisms.

To determine how the cluster of PxxP motifs influences the ability of c-Abl to regulate filopodium formation, we first compared the kinase activity between c-Abl and the 1234 mutant by *in vitro* kinase assay using GST-CTD as a substrate (Baskaran et al., 1993), but did not detect any significant difference in catalytic activity between c-Abl and 1234 (supplemental material Fig. S1B). Next, we compared the subcellular localization of wt c-Abl and the mutant 1234 in Abl DKO cells. In both spreading and adherent cells, there were no apparent differences in the subcellular distribution of c-Abl vs. the mutant 1234; both proteins are similarly distributed throughout the nucleus, cytoplasm, and plasma membrane (Fig. 1B and data not shown). Thus, we conclude that mutation of all four PxxP motifs does not alter the basal catalytic activity or subcellular localization of Abl.

### Identification of possible interaction partners of Abl PxxP motifs by screening SH3 domain phage display library

Because PxxP motifs are known to interact with SH3 domains, we used a phage display library carrying essentially all known human SH3 domains (Karkkainen et al., 2006) to screen for SH3 domains that preferentially bind to the Abl PxxP motifs. This comprehensive and competitive approach is designed to identify which SH3 domains, out of the entire genomic complement, bind best to a given target protein of interest. As the bait for screening, we used a GST fusion protein encoding Abl residues 534–650. Although this fusion contains only the 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> PxxP motifs, the Abl 1234 and 123 mutants were indistinguishable in the filopodium formation assay (data not shown). The presence of either arginine or lysine in the +6 position of the PxxP motif strongly affects SH3 domain binding specificity (Mayer and Saksela, 2004); therefore, the Abl PxxP motifs were classified accordingly. One class (PxxPxK) includes the 1<sup>st</sup>, 2<sup>nd</sup>, and 4<sup>th</sup> PxxP motifs, and the other (PxxPxR) consists of the 3<sup>rd</sup> PxxP

motif (supplemental material Fig. S1C). To screen for interaction partners of each class of sites, in addition to the wt PxxP bait (construct “WT”), we also used baits with inactivating mutations of PxxP motifs 1 and 2 (construct “12”) or motif 3 (construct “3”).

When the wt fusion or the fusion containing only the 1<sup>st</sup> and 2<sup>nd</sup> functional PxxP motifs (construct “3”) of Abl was used, the 1<sup>st</sup> (N-terminal) SH3 domain of Crk was the predominant binder identified (Table 1). This agrees with previous findings that Crk can bind to Abl PxxP motifs (Feller et al., 1994; Ren et al., 1994), and that the N-terminal SH3 of Crk prefers lysine-containing binding motifs (Feller, 2001). When SH3 domains of Crk and CrkL, the second member of the Crk family of adaptors (ten Hoeve et al., 1993), were omitted from the library, no SH3 domains were selected using the 1<sup>st</sup> and 2<sup>nd</sup> PxxP motifs, suggesting that these motifs are very selective for Crk and CrkL SH3 domains (data not shown). For the fusion expressing only the 3<sup>rd</sup> functional Abl PxxP motif (construct “12”), more diverse SH3 domains were found to bind (Table 1). These include the 2<sup>nd</sup> SH3 domain of Nck1, which belongs to the Nck family of adaptor proteins comprised of Nck1 and Nck2 (Braverman and Quilliam, 1999) and the 3<sup>rd</sup> SH3 domains of Ponsin/CAP and ArgBP2, which belong to the SoHo family of proteins comprised of Ponsin, Vinexin, and ArgBP2 (Kioka et al., 2002). Somewhat surprisingly, the 3<sup>rd</sup> Abl PxxP motif also selected the 1<sup>st</sup> SH3 domain of Crk and of CrkL, albeit not as dominantly as the 1<sup>st</sup> and 2<sup>nd</sup> PxxP motifs. Among these proteins, it has been previously shown that fibroblasts with inactivation of Nck family genes have decreased filopodium formation during attachment (Woodring et al., 2004). The Crk and SoHo families of proteins have been shown to bind to PxxP motifs of c-Abl (Lin et al., 2001; Mitsushima et al., 2006; Ren et al., 1994), but the significance of such interactions in the regulation of filopodium formation during attachment is unknown.

### **Nck and Crk family adaptor proteins bind to c-Abl PxxP motifs and regulate filopodium formation during attachment**

To examine effects of candidate binding proteins of Abl PxxP motifs on filopodium formation, each candidate was overexpressed in NIH3T3 fibroblasts using a retroviral vector carrying EGFP as a marker (supplemental material Fig. S2A and D). In addition to the candidate proteins found by library screening, Grb2 and Abi family proteins were also overexpressed, since they have been previously shown to interact with the PxxP motifs of Abl (Dai and Pendergast, 1995; Ren et al., 1994; Shi et al., 1995). Filopodium formation of cells overexpressing each candidate was then observed during attachment to fibronectin (supplemental material Fig. S2B and E). Among the candidates tested, only Nck1 and Nck2 overexpression increased filopodium formation significantly (supplemental material Fig. S2B, C, E and F, and Movie 2). These filopodia looked longer and more slender than those induced by c-Abl in Abl DKO cells. The slight difference in appearance is most likely due to differences between NIH3T3 cells and Abl DKO cells. Also, the increased number and length of filopodia induced by Nck2 compared to Nck1 is likely due to higher expression of Nck2 (compare to Fig. 2B and E, where less Nck2 is expressed). In contrast to Nck family adaptors, overexpression of Crk family adaptors, CrkII and CrkL, decreased filopodium formation while enhancing lamellipodium formation (supplemental material Fig. S2B, C, E, and F, and Movie 2). While it was statistically insignificant, we also consistently observed a decrease in filopodia in cells overexpressing another Crk splicing variant, CrkI (supplemental material Fig. S2B and C). All other candidate proteins had no obvious impact on the number of filopodia in spreading cells (supplemental material Fig. S2D, E, and F).

Based on these results, we further investigated the Crk and Nck families of adaptors as candidate effectors of the Abl PxxP motifs. Both of these families have two closely related members that consist largely of SH2 and SH3 domains (supplemental material Fig. S1A). For the Crk family members, similarities in their primary structures and specificities of interaction

partners suggest functional redundancy (Feller, 2001). For this reason, CrkII was used in overexpression experiments as a representative of the Crk family of proteins. Likewise, functional redundancy between Nck1 and 2 has also been supported by several studies (Bladt et al., 2003; Rivera et al., 2006). Because Nck1 was difficult to overexpress by retroviral infection, we used Nck2 in overexpression experiments.

To confirm the involvement of Crk and Nck families of proteins in regulation of filopodium formation during attachment, we used siRNA to knock down Crk family (CrkI/II and CrkL) and Nck family (Nck1 and 2) adaptor proteins in NIH3T3 cells (Fig. 2A). Using these siRNA target sequences, we were able to knock down approximately 90% of CrkI, 85% of CrkII, 99% of CrkL, and 75% of Nck1/2 throughout the experiments. Knockdown of Crk family adaptors increased filopodium formation during attachment, in contrast to overexpression which resulted in decreased filopodia (Figure 2B and C and Movie 3). Knockdown of Nck family adaptors decreased filopodium formation while the overexpression of Nck family adaptors increased filopodia (Figure 2B and C).

To test whether Crk and Nck family adaptors regulate filopodium formation through a c-Abl mediated pathway or not, NIH3T3 cells overexpressing Nck2 or CrkII were treated with the Abl catalytic inhibitor, STI-571 (Imatinib) (Druker et al., 1996) (Fig. 2D). We found that STI-571 treatment significantly reduced filopodium formation in spreading cells overexpressing Nck2 (Fig. 2E and F). Even though cells overexpressing CrkII showed very few filopodia, treating these cells with STI-571 further reduced filopodium formation (Fig. 2E and F). These data strongly suggest that these adaptors are the critical downstream effectors of c-Abl PxxP motifs to regulate filopodium formation.

By GST-pulldown and overlay assays, we also confirmed that Nck1 directly binds to the 3<sup>rd</sup> and CrkII to the 1<sup>st</sup>, 2<sup>nd</sup>, and 4<sup>th</sup> PxxP motifs of Abl, respectively (supplemental material Fig. S1D and E). Although the phage display library screening suggested that interaction between the 1<sup>st</sup> Crk SH3 domain and the 3<sup>rd</sup> PxxP motif of Abl can occur, this interaction was not detected by overlay or GST-pulldown assays (supplemental material Fig. S1D and E). Abl variants with mutations either in the Crk binding sites (124), or the Nck binding site (3), induced fewer filopodia during attachment than wt c-Abl, and the effects of these mutations was additive (Fig. 1C). These data all suggest that direct interaction of c-Abl via its PxxP motifs with both Crk and Nck adaptors is important for the ability of Abl to regulate filopodium formation during attachment.

### Functional roles of PxxP motifs for regulation of filopodia during attachment

To further investigate the mechanisms whereby Abl regulates filopodium formation via its PxxP motif binding partners, we tested whether the PxxP motifs are involved in tyrosine phosphorylation of Abl substrates during attachment or not. Previously, it has been shown that phosphorylation of Dok1 at Y361 by c-Abl increases filopodium formation (Woodring et al., 2004). Thus, we examined Dok1 tyrosine phosphorylation in NIH3T3 cells overexpressing c-Abl or a series of PxxP motif mutated forms of Abl. Strikingly, overexpression of Abl variants lacking the Nck binding site (mutants 3 and 1234) did not affect phosphorylation of Dok1, while overexpression of wt c-Abl or the variant lacking Crk binding sites (124) dramatically increased Dok1 phosphorylation (supplemental material Fig. S3A). However, Nck family knockdown did not reduce tyrosine phosphorylation of Dok1 during attachment (data not shown). Thus while Abl/Nck interaction may be sufficient for Dok1 phosphorylation during attachment, it does not appear to be necessary.

Next, as also shown in previous studies, we showed that Crk itself was tyrosine phosphorylated by c-Abl during attachment ((Kain and Klemke, 2001), supplemental material Fig. S3B). CrkII phosphorylation has been proposed to promote intramolecular interaction between the CrkII

SH2 domain and the phosphorylated Y221 peptide (Kain and Klemke, 2001; Kobashigawa et al., 2007). This intramolecular interaction prevents other proteins from binding to the SH2 and SH3 domains of CrkII and blocks CrkII-mediated signaling. The Crk binding sites of Abl (PxxP motifs 1, 2, and 4) were required for c-Abl to phosphorylate CrkII during attachment (supplemental material Fig. S1B and S3C), and Abl carrying mutations in the CrkII binding sites (124) did not increase filopodium formation as efficiently as c-Abl (Fig. 1C). Furthermore, overexpression of CrkII suppressed filopodium formation. Conversely, Crk family knockdown cells increased filopodium formation during attachment (Fig. 2). Taken together, these results imply that c-Abl uses PxxP motifs 1, 2, and 4 to interact with and phosphorylate Crk, thereby inhibiting Crk signaling during attachment and leading to increased filopodium formation in spreading cells.

Previously, it has been shown that interaction of CrkII with its critical SH2 domain binding partner, p130Cas, is required for an increase in motility, and also that c-Abl disrupts the CrkII-p130Cas complex during attachment (Kain and Klemke, 2001). Thus, we tested whether CrkII-p130Cas complex formation regulates filopodium formation during attachment by comparing fibroblasts lacking p130Cas (p130CasKO) with ones re-expressing p130Cas. p130Cas KO cells formed more filopodia than p130Cas KO cells re-expressing p130Cas during attachment (supplemental material Fig. S4A, B, and C). This implies that one of the mechanisms whereby Abl increases the number of filopodia during attachment is the disruption of an interaction between CrkII and p130Cas.

### **c-Abl via its PxxP motifs suppresses activation of Rac1 and controls the balance between filopodia and lamellipodia during attachment**

Activation of Cdc42 and Rac1 small GTPases is sufficient for the formation of filopodia and lamellipodia, respectively, and formation of these actin structures is often achieved by the activation of these small GTPases (Ridley, 2006). Previously, c-Abl has been shown to block lamellipodium formation at the leading edge of cells by tethering Rac1 to dorsal membrane ruffles during attachment without affecting the activity of Rac1 (Jin and Wang, 2007; Woodring et al., 2002). In this study, we revisited the question of the influence of c-Abl on the activity of small GTPases during attachment. When c-Abl was overexpressed in spreading NIH3T3 cells, resulting in increased filopodium formation and decreased lamellipodium formation, we found that Rac1 activation was reduced compared to control cells during attachment (Fig. 3A). This is inconsistent with the previous report, where c-Abl did not influence Rac1 activity during attachment. This may be due to differences in the experimental approaches, i.e. c-Abl overexpression vs. pharmacological inhibition, as we confirmed that Rac1 activity in the presence of the same catalytic inhibitor of c-Abl was indistinguishable from control cells (Fig. 3B). These results suggest that c-Abl can negatively regulate Rac1 activation during attachment, though the contribution of endogenous c-Abl probably represents a relatively small portion of the total pool of activated Rac1.

We next addressed whether the PxxP motifs are involved in this c-Abl-mediated regulation of Rac1 activity during attachment. When we overexpressed in NIH3T3 cells the PxxP motif-mutated form of Abl, 1234 (which did not promote filopodium formation as efficiently as wt c-Abl), the level of Rac1 activation was similar to that in control cells (Fig. 3A). This indicates that c-Abl requires its PxxP motifs to reduce Rac1 activation during attachment. Furthermore, the downstream effectors, Nck and Crk, were examined by overexpression and knockdown experiments. Like c-Abl, overexpression of Nck2 in NIH3T3 cells (which resulted in increased filopodium formation and decreased lamellipodium formation) decreased Rac1 activation compared with control (Fig. 3A). However, Nck family knockdown did not affect Rac1 activation (Fig. 3B). This is similar to the finding that inhibition of endogenous c-Abl did not affect Rac1 activation during attachment, suggesting that the contribution of endogenous Nck

family adaptors represents a relatively small portion of the total pool of activated Rac1 (Fig. 3B). On the other hand, CrkII overexpression, which resulted in increased lamellipodium formation and decreased filopodium formation, enhanced Rac1 activation (Fig. 3A). Knockdown of the Crk family, which resulted in increased filopodium formation and decreased lamellipodium formation, blunted Rac1 activation upon attachment (Fig. 3B). In contrast to the various effects on Rac1 activity, we could not observe significant changes in Cdc42 activation during attachment in any of the contexts examined here (Fig. 3A and B), though Cdc42 activity was modestly enhanced in suspended cells overexpressing Nck2 or c-Abl (Fig 3A).

Taken all together, our small GTPase results show that c-Abl regulates the balance between filopodium and lamellipodium formation during attachment by negatively regulating Rac1 activity, without affecting total Cdc42 activity, through the interaction of its PxxP motifs with Crk and Nck.

### **c-Abl, Nck, and Crk regulate cell surface area during attachment**

Next, we asked how filopodium and lamellipodium formation relate to the spreading behavior of cells on fibronectin-coated surfaces. To address this question, we observed the attachment of NIH3T3 cells overexpressing c-Abl or Nck2 to enhance filopodium formation, or CrkII to enhance lamellipodium formation. At 10 minutes after replating onto fibronectin, when filopodia or lamellipodia are robustly forming, NIH3T3 cells overexpressing c-Abl, Nck2, or CrkII showed significant differences in the average surface area occupied by the cell (Fig. 4A and B, and Movie 2). Cells overexpressing c-Abl or Nck2 had a smaller average cell area and greater numbers of filopodia (Fig. 4A and B). On the other hand, cells overexpressing CrkII, which enhanced lamellipodium formation, had a larger average cell area (Fig. 4A and B). We also compared the average cell area for Abl DKO cells expressing various forms of Abl 10 minutes after replating onto fibronectin. Cells re-expressing wt c-Abl had a smaller average cell area compared to parental Abl DKO cells or those re-expressing the 1234 or KD mutants, which do not enhance filopodium formation (Fig. 4C and D, Movie 1). These results indicate that c-Abl regulates the surface area of the cell during attachment through its PxxP motifs.

To further confirm the effect of filopodium and lamellipodium formation on cell spreading, cells treated with STI-571, and Nck or Crk family knockdown NIH3T3 cells were examined. We found that cells treated with STI-571 or Nck family knockdown cells, where filopodium formation was reduced, had increased average cell area 9 minutes after replating (Fig. 4E and 4F). On the other hand, Crk family knockdown cells, which have more numerous filopodia, had decreased cell area 10 minutes after replating (Fig. 4G and H, Movie 3). Also, p130Cas KO cells had increased filopodium formation and decreased cell area 10 minutes after replating, compared with KO cells re-expressing p130Cas (supplemental material Fig. S4D and E). Note that all cells examined here eventually attained a similar cell area at 20 minutes (Movies 1, 2, and 3). Spreading kinetics from live images (Movies 1, 2, and 3) and our quantitative cell area assay suggest that c-Abl controls the balance between filopodium and lamellipodium formation and thereby regulates the speed of cell spreading during early attachment through the interaction of its PxxP motifs with Nck and Crk family adaptor proteins.

### **c-Abl regulates focal adhesion formation through the interactions of PxxP motifs with Crk and Nck during attachment**

Formation of focal adhesions is important for productive spreading and adhesion of cells during attachment. Thus, focal adhesion formation during attachment was examined in NIH3T3 cells overexpressing c-Abl, 1234, Nck2, or CrkII. To quantify focal adhesion formation, the phosphorylation levels of Fak Y397 and Paxillin Y118 were detected by western blotting. These proteins are building blocks of focal adhesions, and their phosphorylation are well-



known indicators of focal adhesion formation. In comparison with control cells, Fak and Paxillin phosphorylation after attachment on a 2  $\mu\text{g}/\text{ml}$  fibronectin coated-surface was reduced in NIH3T3 cells overexpressing c-Abl or Nck2, which had increased filopodia and spread slowly, and increased in cells overexpressing CrkII, which had increased lamellipodia and spread rapidly (Fig. 5A). Unlike c-Abl, the 1234 mutant did not suppress phosphorylation of Fak and Paxillin under these conditions (Fig. 5A). Thus c-Abl and Nck negatively regulate focal adhesion formation during attachment, while Crk positively regulates focal adhesion formation. Importantly, c-Abl uses its PxxP motifs for this negative regulation. When fibronectin was increased to 6  $\mu\text{g}/\text{ml}$ , differences in the level of Fak and Paxillin phosphorylation were no longer apparent, suggesting that other signaling pathways contribute to focal adhesion formation at very high signal levels.

We also assayed Fak and Paxillin phosphorylation levels in cells treated with STI-571, and in Nck or Crk family knockdown cells. Crk family knockdown cells, in which filopodium formation was enhanced, showed reduced Fak and Paxillin phosphorylation during attachment (Fig. 5B). However, Nck knockdown or inhibition of Abl did not affect their phosphorylation levels compared to control cells (Fig. 5B). This is discussed later in this section.

In addition to quantitative differences in focal adhesion formation, we also examined qualitative differences by immunofluorescence staining of vinculin (a building block and marker of focal adhesions) in NIH3T3 cells overexpressing c-Abl, Nck2, or CrkII during attachment. Control cells displayed dispersed and prominent focal adhesions close to the leading edge (supplemental material Fig. S5A). In agreement with our result of Fak and Paxillin phosphorylation, cells overexpressing c-Abl or Nck2, which had increased filopodia and spread slowly, did not form prominent focal adhesions at the leading edge (supplemental material Fig. S5A). In contrast, cells overexpressing CrkII, which had increased lamellipodia and spread rapidly, formed prominent and continuous focal adhesions at the leading edge (supplemental material Fig. S5A). We also tested the impact of c-Abl and its PxxP motifs in the extent and pattern of focal adhesion formation in Abl DKO cells. Re-expression of c-Abl in Abl DKO cells disrupted the prominent and continuous focal adhesions at the leading edge observed in control Abl DKO cells (supplemental material Fig. S5B), consistent with a previous report (Woodring et al., 2002). Abl DKO expressing 1234 formed prominent and continuous focal adhesions at leading edge of cells, similar to control Abl DKO cells and to those expressing the KD mutant (supplemental material Fig. S5B).

Furthermore, the pattern and extent of focal adhesions were examined in STI-571 treated, or Nck or Crk family knockdown cells. As expected, STI-571 treated and Nck family knockdown cells, which exhibited reduced filopodium formation and rapid cell spreading, displayed prominent and continuous focal adhesions at the leading edge (supplemental material Fig. S5C). However, the total amount of prominent focal adhesions was similar to the control cells. This could explain why the phosphorylation levels of Fak and Paxillin in STI-571 treated or Nck family knockdown cells did not change when compared to the control cells (Fig. 5B). In contrast, Crk family knockdown cells, characterized by increased filopodium formation and slow spreading speed, did not form discrete focal adhesions at the leading edge during attachment (supplemental material Fig. S5D). Moreover, when compared to p130Cas KO cells, the re-expression of p130Cas in the KO cells resulted in reduced filopodium formation and more prominent focal adhesions on the leading edge of cells (supplemental material Fig. S4F).

Taken together, these results suggest that formation of filopodia and lamellipodia are strongly correlated with focal adhesion formation, and that c-Abl inhibits focal adhesion formation during attachment through pathways mediated by the Crk and Nck families of adaptor proteins.

## Discussion

This study demonstrates that the c-Abl PxxP motifs play an essential role in regulating cell spreading and focal adhesion formation during attachment. Through interactions of its PxxP motifs with downstream effectors, c-Abl inhibits Rac1 activation and modulates the balance between filopodium and lamellipodium formation. We use a combination of mutational analysis, pharmacological inhibition and perturbation of protein levels by silencing and overexpression to demonstrate direct roles for the Nck and Crk families of SH2/SH3 adaptors in this process. We show that inhibition of Crk signaling by Abl-dependent phosphorylation, and positive signaling through Nck engagement, constitute the molecular mechanisms by which the cluster of c-Abl PxxP motifs inhibits lamellipodium and focal adhesion formation, and promotes formation of filopodia during cell attachment.

We showed that c-Abl interacts with Crk through its 1<sup>st</sup>, and 2<sup>nd</sup>, and 4<sup>th</sup> PxxP motifs and facilitates phosphorylation of tyrosine 221 of CrkII. This phosphorylation is known to induce an intramolecular interaction that impairs the interaction of its SH2 and SH3 domains with other proteins, and consequently inhibits Crk signaling (Abassi and Vuori, 2002; Kain and Klemke, 2001; Kobashigawa et al., 2007). The inhibition of CrkII signaling by c-Abl is also reported by others in several biological situations (Kain and Klemke, 2001; Noren et al., 2006). To elucidate the inhibitory effect of Crk signaling, we explored the roles of Crk during attachment, which were found to promote lamellipodium formation through Rac1 activation and focal adhesion formation. These phenotypes are probably regulated by the interactions of Crk with p130Cas and Paxillin (SH2 domain), and Dock180 (N-terminal SH3 domain) since the formation of p130Cas-CrkII-Dock180 or p130Cas- and Paxillin-CrkII complexes have been proposed to promote lamellipodium formation (Albert et al., 2000) and assembly of focal adhesions (Abassi and Vuori, 2002; Birge et al., 1993; Kira et al., 2002; Richardson et al., 1997; Sakai et al., 1994), respectively. Our finding that spreading behavior is altered in p130Cas KO cells also supports this hypothesis. Thus we propose that following CrkII phosphorylation by c-Abl during attachment, the disruption of CrkII interactions with its binding partners leads to inefficient formation of focal adhesions and suppression of Rac1 activation resulting in decreased lamellipodium formation. These cause increased filopodium protrusions and eventually slow cell spreading. In addition to this pathway, another group has shown that c-Abl regulates cell attachment through a pathway involving CrkII and dynamin by blocking the localization of Rac to the leading edge of cells (Jin and Wang, 2007). This suggests that c-Abl can regulate cell spreading through multiple pathways mediated by Crk.

We also found that c-Abl regulates cell attachment through the interaction of the 3<sup>rd</sup> PxxP motif with Nck family adaptors. In contrast to the interaction with Crk, this interaction promotes downstream Nck signaling, as the effects are enhanced by Nck overexpression and attenuated by Nck knockdown. Despite our efforts, the downstream targets of the Nck-Abl complex during attachment were not identified in this study. Several possibilities warrant further study, however. The binding of membrane-targeted Nck SH3 domains has been shown to increase the catalytic activity of c-Abl (Smith et al., 1999). Thus, direct Nck-c-Abl interaction at the membrane may locally increase the catalytic activity of c-Abl to enhance filopodium formation. Furthermore, the activation of N-WASP by c-Abl-dependent phosphorylation may contribute to the elongation of filamentous actin and formation of filopodia (Burton et al., 2005; Torres and Rosen, 2003). Since Nck has multiple SH3 domains that interact with both c-Abl and N-WASP (Rivero-Lezcano et al., 1995; Rohatgi et al., 2001), it is tempting to speculate that Nck may enable c-Abl to phosphorylate N-WASP more efficiently by physically tethering the two proteins. In addition, our silencing and overexpression experiments show that Nck negatively regulates lamellipodium extension by inhibiting Rac1 activation and formation of focal adhesions during attachment. Elucidation of the molecular mechanisms by which Nck

contributes to c-Abl-dependent regulation of the cytoskeleton and focal adhesion formation during cell attachment awaits further investigation.

Of the two Abl family kinases, this study solely focused on c-Abl. c-Abl and Arg are known to function redundantly in many cases. Intriguingly, it was shown that Arg phosphorylated p190RhoGAP and inhibited focal adhesion formation during attachment (Hernandez et al., 2004; Peacock et al., 2007), while c-Abl did not phosphorylate p190RhoGAP, and fibroblasts lacking c-Abl had normal focal adhesions (Hernandez et al., 2004; Peacock et al., 2007; Woodring et al., 2004). These findings suggest that c-Abl cannot compensate for Arg in this specific situation. However, in our study we show that overexpression of c-Abl clearly has the ability to inhibit focal adhesion formation. This apparent contradiction might be due to a difference in experimental approaches, i.e. loss of protein vs. overexpression. Furthermore, we observed that re-expression of Arg in Abl DKO cells promoted filopodium formation during attachment similarly to c-Abl re-expression (data not shown). Thus, these data suggest that c-Abl and Arg can redundantly function during attachment. Most importantly, Arg shares the same consensus sequences with c-Abl at the 1<sup>st</sup> and 2<sup>nd</sup> (PxxPxK) and 3<sup>rd</sup> (PxxPxR) PxxP motifs. This suggests that Arg also interacts with Crk and Nck through its PxxP motifs and regulates cell spreading and focal adhesion formation.

In light of the results presented in this study, we propose a model for the functional role of c-Abl during the early stages of cell spreading (Fig. 6). In suspended cells (inactive c-Abl), the PxxP motifs 1, 2, and 4 of c-Abl are sequestered by CrkII and this prevents the 3<sup>rd</sup> PxxP motif from interacting with Nck. Upon integrin engagement during attachment, c-Abl becomes activated, the bound CrkII is phosphorylated and signaling mediated by this protein is inhibited. The release of CrkII from the PxxP motifs 1, 2, and 4 then allows the recruitment of Nck to the 3<sup>rd</sup> PxxP motif. Both Crk inactivation and Nck recruitment suppress the protrusion of lamellipodia and the assembly of focal adhesion, resulting in increased formation of filopodia and slower cell spreading.

Our findings suggest that through the interaction of its PxxP motifs with Crk and Nck adaptors, c-Abl fine-tunes the local actin organization of cells as they react to their surrounding environment. Interactions mediated by the Abl PxxP motifs are likely to also play a critical role in other biological processes where c-Abl, Crk, and Nck regulate actin dynamics, such as neuronal development and pathogen invasion (Ballif et al., 2004; Burton et al., 2003; Fawcett et al., 2007; Koleske et al., 1998). Thus, insights gained from the present studies on cell attachment are likely to be highly relevant to the molecular mechanisms underlying such diverse and important activities

## Materials and Methods

### Antibodies and Reagents

Antibodies directed against Abl (8E9, BD Pharmingen; K-12, Santa Cruz Biotechnology; OP-20, Calbiochem), CrkI/II (BD Transduction Laboratories), CrkL (C-20, Santa Cruz), Nck1/2 (BD Transduction Laboratories), phosphotyrosine, pTyr (P-Tyr-100, Cell Signaling Technology), Dok1 (A3 and M276, Santa Cruz Biotechnology), Vinculin (VIN-11-5, Sigma-Aldrich), pY397-Fak (44-624G, Invitrogen), Fak (C-20, Santa Cruz), pY118-Paxillin (Cell Signaling), Paxillin (BD Transduction Laboratories), p130Cas (BD Transduction Laboratories), Rac1 (23A8, Millipore), Cdc42 (BD Transduction Laboratories), FLAG (M2, Sigma-Aldrich), EGFP (FL, Santa Cruz Biotechnology), and Actin (KJ43A, Sigma-Aldrich) were purchased from commercial sources. Antibodies recognizing Abi1 and 2, Ponsin, and Vinexin- $\alpha$  and - $\beta$  were the generous gifts from Ann Marie Pendergast, Yoshimi Takai, and Noriyuki Kioka, respectively. Polyclonal rabbit antibody to Grb2 was previously described (Gupta and Mayer, 1998). STI-571 was generously provided by Novartis.

## Plasmids

The cDNAs encoding wt and kinase-defective (KD) mouse type IV c-Abl, human Nck1 and 2 and Grb2, and rat p130Cas were previously described (Jackson and Baltimore, 1989; Mayer et al., 1995; Rivera et al., 2006). The cDNA encoding human Abi1 and Abi2 (Ann Marie Pendergast), human CrkI and II (Michiyuki Matsuda), human CrkL (John Groffen), mouse Ponsin (Yoshimi Takai), and human Vinexin- $\alpha$  and - $\beta$  (Noriyuki Kioka) were generous gifts. A series of Abl PxxP motif mutants, in which amino acids PxxP were changed to AxxA, an Abl SH2 domain mutant, SH2\*, in which arginine 171 was changed to lysine, and constitutively activated form of Abl, PP, (Barila and Superti-Furga, 1998), were generated by polymerase chain reaction-based mutagenesis using Pfu DNA polymerase (Stratagene). Various Abl cDNAs were inserted into pEBB, pMSCV-puro, and pMIGR-1 vectors (Pear et al., 1998; Tanaka et al., 1995). p130Cas cDNA was inserted into pMSCV-puro vector. All other cDNAs were inserted into pMIGR-1 vector. pET-GST-Pak1-PBD for GTPase assay, pGEX-CTD for in vitro kinase assay, and pGEX-Crk-225 for in vitro kinase assay and far-western blotting were obtained from Dan Wu, Jean Wang, and Stephan Feller, respectively (Baskaran et al., 1993; Kharbanda et al., 1995). GST-Nck SH3s construct for far-western blot probe was generated by inserting 1<sup>st</sup> and 2<sup>nd</sup> SH3 domains of human Nck1 into pGEX-6P-1 vector (GE Healthcare). GST-wt, 12, and 3 Abl constructs for baits of phage display library screening were generated by inserting amino acid residues 534-650 of wt c-Abl, 12, and 3 mutant Abl into pGEX-6P-1 vector. Short hairpin RNAs (shRNA) targeting mouse CrkI/II/L, Nck1, or Nck2 were inserted into pSUPER.retro vector (Oligoengine). The sequences for shRNA knockdown constructs targeting mouse CrkI/II/L (generous gift from Susan Veals and Jonathan Cooper), Nck1, or Nck2 were: 5'-gggatgattcctgtccctta-3' (CrkI/II/L); 5'- gaatcttcgccaatgatt-3' (Nck1); and 5'- ggaggagcttagtttcgagaa-3' (Nck2), respectively.

## Cell culture, transfection, viral infection, and fibronectin stimulation of cells

Abl and Arg double knockout mouse embryonic fibroblasts (Abl DKO MEFs), and p130Cas knockout mouse embryonic fibroblasts (p130Cas KO MEFs) were generous gifts from Tony Koleske and Amy Bouton, respectively. Abl DKO MEFs, p130Cas KO MEFs, and 293T human embryonic kidney cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Mediatech) containing 10% (v/v) fetal bovine serum (Gemini Bio-products) and 1x Penicillin-Streptomycin solution (Mediatech). NIH3T3 cells were maintained in DMEM containing 10% (v/v) bovine serum (Gemini Bio-products) and 1x Penicillin-Streptomycin solution. For viral production, 293T cells were transfected with retroviral vector plus packaging plasmids pMD.env and pMD.gag.pol by a calcium phosphate method, and medium containing virus was harvested. NIH3T3 or Abl DKO cells were infected with virus in the presence of 2  $\mu$ g/ml polybrene (Millipore). NIH3T3 infected with pMIGR-1 vector-derived virus were replated and serum-starved 24 hours post-infection for later experiments. NIH3T3 cells infected with pSUPER vector-derived viruses were replated and serum-starved 72 hours post-infection. Abl DKO or p130Cas KO cells infected with pMSCV-puro derived virus were drug-selected with 1.0  $\mu$ g/ml puromycin (Sigma-Aldrich) for 7 days, recovered for 7 days without drug, and kept for further experiments up to 5 weeks. Preparation of suspended cells and attachment to fibronectin-coated surfaces was performed as previously described (Woodring et al., 2001). For STI-571 treatment, 3  $\mu$ M STI-571 was continuously maintained during and after serum starvation until the cells were fixed or harvested.

## Biochemistry

Cells were lysed with kinase lysis buffer (25 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM Na<sub>3</sub>VO<sub>4</sub> 10 mM sodium pyrophosphate, 10 mM  $\beta$ -glycerophosphate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1  $\mu$ g/ml aprotinin). Anti-Dok1 (M-276), -Abl (OP-20, in vitro kinase assay), and -Abl (K-12, far-

western blotting) antibodies were used for immunoprecipitation. For far-western blotting, probes were prepared by pre-incubation of 5  $\mu\text{g}/\text{ml}$  GST or GST fusion protein with 1.25  $\mu\text{g}/\text{ml}$  GSH-HRP (Sigma-Aldrich) in PBS for 1 hour at room temperature, and further procedures were performed as previously described (Nollau and Mayer, 2001). In vitro kinase and GTPase assays were performed as previously described (Azim et al., 2000; Pluk et al., 2002). The construction of phage display library and screening method were previously described (Karkkainen et al., 2006). For phage display, GST-wt, -12, and -3 Abl were used as baits, and after the 1st round of selection, the positive colonies were sequenced.

## Microscopy

Cells were fixed on fibronectin-coated glass coverslips by adding one-tenth total culture medium volume of 37% (w/v) formaldehyde into a culture dish for 5 minutes, and permeabilized with PBS containing 0.5% (v/v) NP-40 for 5 minutes. The coverslips were blocked with PBS containing 1% (w/v) BSA for 1 hour at room temperature. Fixed cells were then stained with either anti-Vinculin or anti-Abl (8E9) antibodies followed by goat-anti-mouse secondary antibody conjugated with either Alexa-fluor 488 or 647 (Invitrogen), phalloidin conjugated with Texas-red (Invitrogen), and Hoechst 33342 (Sigma-Aldrich). Photomicrographs were obtained on a Zeiss 510 laser-scanning confocal microscope. For live cell imaging, coverslips were placed into an Attoflour chamber (Invitrogen) prior to the addition of cells. After adding cell suspension to the chamber, images were taken by differential interference contrast (DIC) microscopy using a Zeiss 410 confocal microscope. For all images, a Zeiss AchroStigmat 40x and N.A.=1.3 oil immersion objective lens was used. Filopodia were quantified by a previously described microspike assay (Woodring et al., 2002) using a slightly modified filopodium definition. Briefly, under phase-contrast microscopy, a thin protrusive structure that projected more than 5  $\mu\text{m}$  from the cell periphery was counted as a filopodium. For spreading assay, an image of phalloidin- and DNA-stained cells was analyzed using NIH ImageJ software (NIH Image), and the average cell area in  $\mu\text{m}^2$  was quantified for each cell type.

## Statistical Analysis

Statistical significance for filopodium number and cell area, and cells with or without filopodia, were determined by ANOVA followed by Tukey's test and chi-square analysis, respectively. All statistical values were obtained from three independent experiments.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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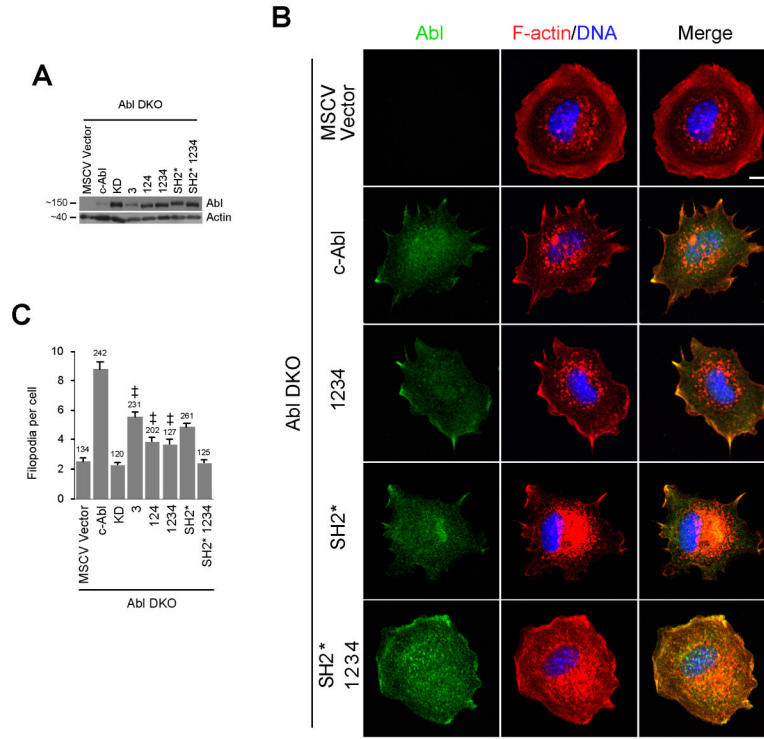
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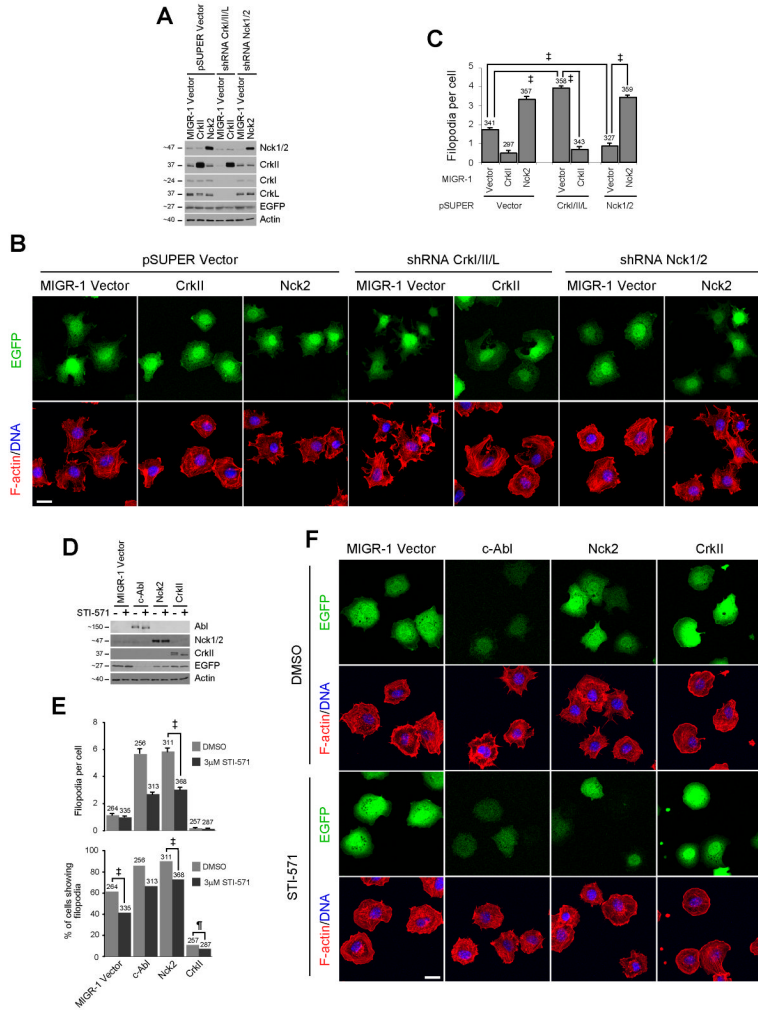
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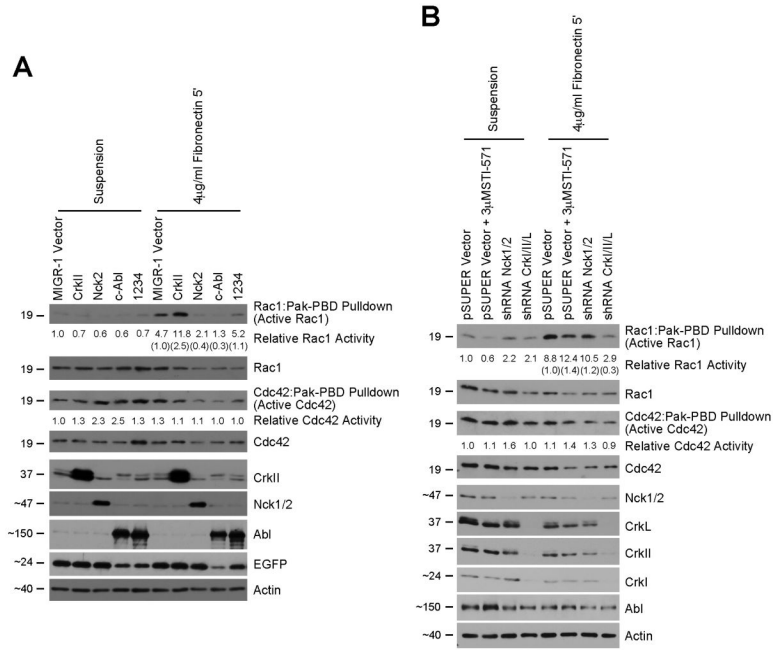




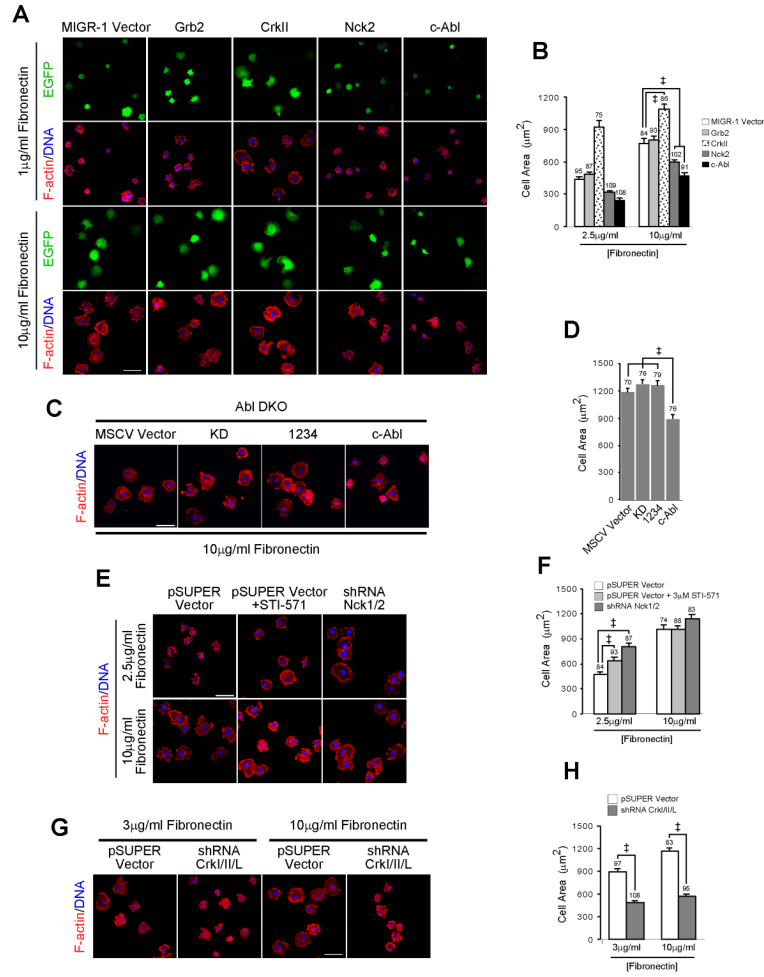
**Fig. 1.** Involvement of Abl PxxP motifs in regulating filopodium formation during attachment. **A.** Expression of c-Abl and variants in Abl DKO cells detected by western blotting with anti-Abl antibody (K-12, recognizing a.a. 521-531 in kinase domain (Woodring et al., 2001)) which binds equally to wt and mutated forms of Abl used in this study (data not shown). Numbers on the left side of blots indicate relative molecular mass in kDa. **B.** Serum-starved Abl DKO cells stably expressing various forms of Abl were plated on coverslips coated with 10  $\mu$ g/ml fibronectin, and fixed at 20 minutes. The fixed cells were stained for DNA (blue), F-actin (red), and c-Abl (green) with Hoechst 33342, phalloidin/Texas-red, and anti-Abl (8E9)/Alexa-fluora 488, respectively. White bar = 10  $\mu$ m. **C.** The number of filopodia was counted from the fixed Abl DKO cells. Graph represents mean and standard error of mean (S.E.M.) of filopodia per cell. The numbers over the bars indicate the number of cells examined. ‡ indicates  $P < 0.05$  when compared with Abl DKO cells re-expressing c-Abl.



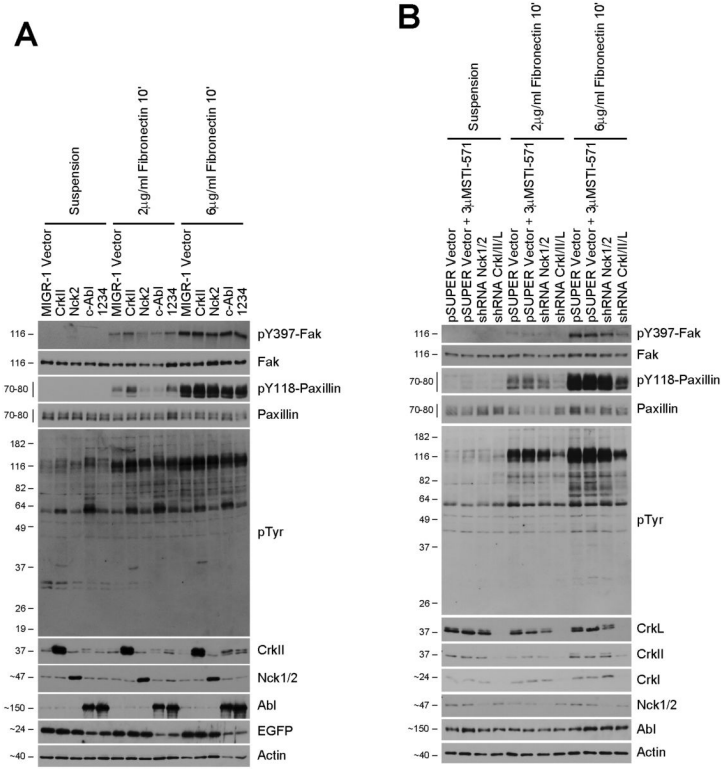
**Fig. 2.** c-Abl regulates filopodium formation during attachment through the interaction of PxxP motifs with Crk and Nck family proteins. **A**, **B**, and **C**. NIH3T3 cells were infected with control retrovirus (derived from pSUPER vector) or the one carrying shRNA sequence targeting CrkI/II/L or Nck1/2. These knockdown cells were super-infected with control retrovirus (derived from MIGR-1 vector) or virus expressing shRNA-resistant (human) CrkII or Nck2. **D**, **E**, and **F**. NIH3T3 cells overexpressing c-Abl, Nck2, or CrkII were treated with or without STI-571. **A** and **D**. Protein expression was examined by western blotting. **B** and **F**. Serum-starved cells were plated on coverslips coated with 10 µg/ml fibronectin, fixed at 20 minutes after plating, and stained for F-actin and DNA. White bars = 20 µm. **C** and **E**. The number of filopodia was counted from the fixed cells. Graph represents mean and/or S.E.M. of filopodia per cell or percentage of cells showing filopodia per total cells. ‡ indicates P<0.05, and ¶ indicates P=0.143.



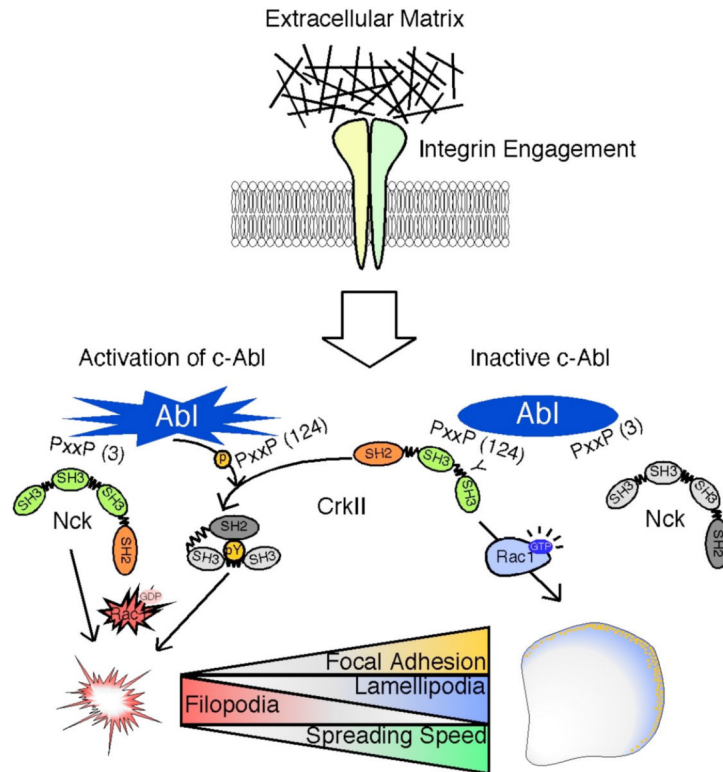
**Fig. 3.** c-Abl and its PxxP motif interaction partners modulate Rac1 activity during attachment. NIH3T3 cells overexpressing CrkII, Nck2, c-Abl or 1234 (**A**), STI-571 treated, or Crk or Nck family knockdown NIH3T3 cells (**B**) were harvested in suspension or 5 minutes after attachment on 4 μg/ml fibronectin-coated dishes. Whole cell lysates (WCL) were subjected to GTPase assay, and the amounts of Rac1 and Cdc42 were detected by western blotting.



**Fig. 4.** c-Abl and its PxxP motif interaction partners regulate the spreading speed of cells. NIH3T3 cells overexpressing Grb2, Nck2, CrkII, or c-Abl (**A** and **B**), Abl DKO cells re-expressing various forms of Abl (**C** and **D**), and Crk family knockdown NIH3T3 cells (**G** and **H**), were plated on fibronectin-coated surface and fixed at 10 minutes when cells were actively spreading. Nck family knockdown or STI-571-treated NIH3T3 cells were fixed at 9 minutes (**E** and **F**). **A**, **C**, **E**, and **G**. Spreading cells were stained for F-actin and DNA. Infected cells express EGFP (green) as marker (**A**). White bar = 50 µm. **B**, **D**, **F**, and **H**. Cell areas for the stained cells were measured. Each bar represents mean and S.E.M. of the cell area. ‡ indicates P<0.05.



**Fig. 5.** c-Abl and its PxxP motif interaction partners regulate focal adhesion formation during attachment. NIH3T3 cells overexpressing CrkII, Nck2, c-Abl or 1234 (**A**), STI-571 treated, or Crk or Nck family knockdown NIH3T3 cells (**B**) were harvested in suspension or 10 minutes after attachment on 2 or 6 μg/ml fibronectin-coated dishes. WCL were subjected to western blotting to detect pY397-Fak or pY118-Paxillin.



**Fig. 6.** Model for roles of Abl, Crk, and Nck in cell spreading. Integrin engagement increases catalytic activity of c-Abl. Due to interaction of Abl PxxP motifs 1, 2, and 4 with CrkII, active c-Abl phosphorylates and inactivates CrkII (colored in grey), together with interaction between PxxP motif 3 and Nck, inhibiting focal adhesion formation and Rac1 activation and thus decreasing lamellipodium formation. These actions also cause increased filopodium formation and eventually slow cell spreading. When c-Abl catalytic activity is low, CrkII remains active and promotes focal adhesion formation and Rac1 activation, resulting in increased formation of lamellipodia. c-Abl and Nck no longer productively interact and transduce signals (Nck under this situation is colored in grey). As a result, low c-Abl activity decreases filopodium formation and accelerates spreading speed of cells.

**Table 1**

SH3 domains binding to Abl PxxP motifs identified by screening a phage display library containing all known human SH3 domains. Details are described in Materials and Methods and (Karkkainen et al., 2006).

Abl PxxP motifs used for bait (Functional PxxP motifs)	Protein bound	SH3 domain (Total # of SH3 domains)	Hit/Total # of Colony Examined
WT (123)	Crk	1st (2)	4/4
12 (3)	Nck1	2nd (3)	2/7
12 (3)	Ponsin	3rd (3)	2/7
12 (3)	ArgBP2	3rd (3)	1/7
12 (3)	Crk	1st (2)	1/7
12 (3)	CrkL	1st (2)	1/7
3 (12)	Crk	1st (2)	6/6