# Induction of cell retraction by the combined actions of Abl–CrkII and Rho–ROCK1 signaling

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Dynamic modulation of cell adhesion is integral to a wide range of biological processes. The small guanosine triphosphatase (GTPase) Rap1 is an important regulator of cell–cell and cell–matrix adhesions. We show here that induced expression of activated Abl tyrosine kinase reduces Rap1-GTP levels through phosphorylation of Tyr221 of CrkII, which disrupts interaction of CrkII with C3G, a guanine nucleotide exchange factor for Rap1. Abl-dependent down-regulation of Rap1-GTP causes cell rounding and detachment only when the Rho–ROCK1 pathway is also activated, for example, by lysophosphatidic acid (LPA). During ephrin-A1–induced retraction of PC3 prostate cancer cells, we show that endogenous Abl is activated and disrupts the CrkII–C3G complex to reduce Rap1-GTP. Interestingly, ephrin-A1–induced PC3 cell retraction also requires LPA, which stimulates Rho to a much higher level than that is activated by ephrin-A1. Our results establish Rap1 as another downstream target of the Abl–CrkII signaling module and show that Abl–CrkII collaborates with Rho–ROCK1 to stimulate cell retraction.

## Introduction

The nonreceptor tyrosine kinase Abl is ubiquitously expressed and is found in both the nucleus and the cytoplasm of mammalian cells. The nuclear Abl kinase is activated upon DNA damage to stimulate apoptosis (Gong et al., 1999; Wang, 2004; Preyer et al., 2007), whereas the cytoplasmic Abl is activated by growth factors, cell adhesion, and bacterial pathogens to regulate F-actin dynamics (Woodring et al., 2002, 2003; Hernandez et al., 2004). Activation of cytoplasmic Abl kinase by platelet-derived growth factor or EGF enhances the activation of the small GTPase Rac and the formation of membrane ruffles (Plattner et al., 1999; Sini et al., 2004). Abl-dependent activation of Rac is also observed upon infection of mouse embryonic fibroblasts with *Shigella flexneri* (Burton et al., 2003). Activated Rac-GTP stimulates actin polymerization through the WASP and WAVE complexes (Eden et al., 2002). Previous studies have shown that Abl also stimulates F-actin microspikes and filopodia through a Rac-independent mechanism (Woodring et al., 2004; Radha et al., 2007), which may involve the direct phosphorylation of the WAVE protein by Abl, leading to the stimulation of actin polymerization (Leng et al., 2005).

Although Abl kinase stimulates actin polymerization, it inhibits cell spreading and cell migration (Frasca et al., 2001; Kain and Klemke, 2001; Jin and Wang, 2007). It has been suggested that Abl kinase inhibits cell migration through tyrosine phosphorylation of CrkII (also known as Crk) to down-regulate Rac-GTP levels (Kain and Klemke, 2001). One of the guanine nucleotide exchange factors (GEFs) for Rac is DOCK180 (Kiyokawa et al., 1998a,b), which is recruited to the plasma membrane through an interaction between CrkII and p130Cas (Kiyokawa et al., 1998a). Phosphorylation of CrkII at tyrosine-221 by Abl (Feller et al., 1994) disrupts the p130Cas–CrkII complex, and may thus account for Abl-dependent down-regulation of Rac-GTP and the inhibition of cell migration (Kain and Klemke, 2001). In breast cancer cells, EphB4 activation by ephrin-B2 causes Abl-dependent CrkII phosphorylation, which is correlated with an inhibition of cell migration and invasion in vitro and tumor growth in vivo (Noren et al., 2006). Recently, tyrosine phosphorylation of CrkII by Abl has also been linked to the dorsal sequestration of Rac-GTP during cell spreading on fibronectin. As a result, Abl promotes dorsal ruffling at the expense of lamellipodia extension to restrain cell spreading

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Abbreviations used in this paper: Doxy, doxycycline; GEF, guanine nucleotide exchange factor; LPA, lysophosphatidic acid; shRNA, small hairpin RNA.

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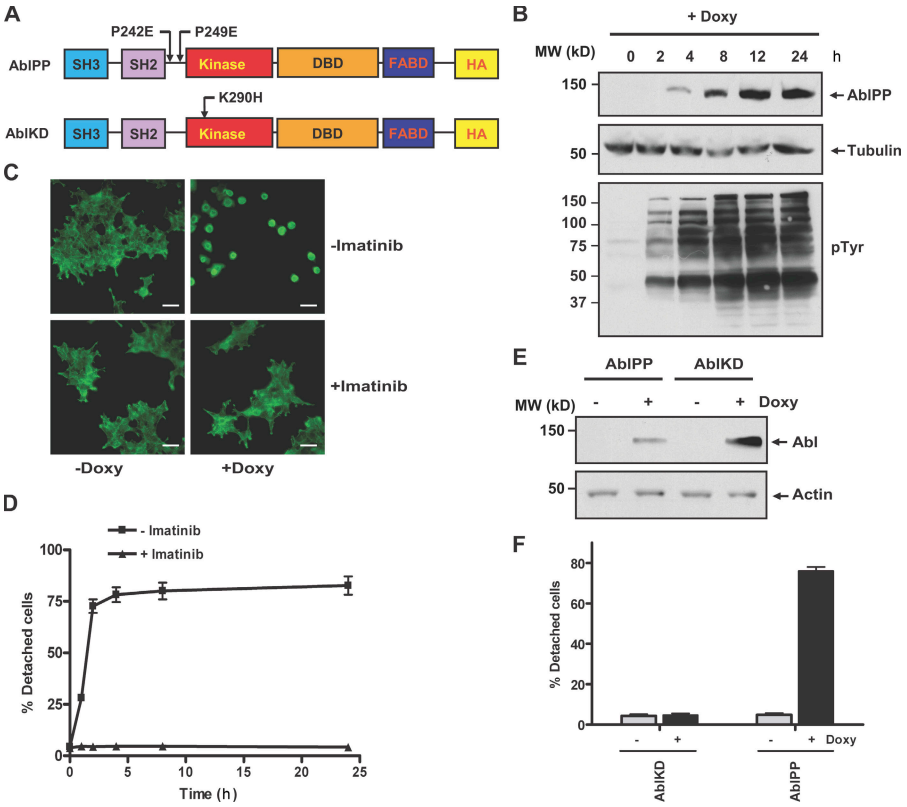


Figure 1. **Induced expression of activated Abl caused kinase-dependent cell detachment.** (A) Diagram of AblPP (murine type IV) and AblKD proteins. SH3, Src homology 3 domain; SH2, Src homology 2 domain; DBD, DNA-binding domain; FABD, F-actin binding domain. The kinase defective mutant (KD) was created by substitution of lysine$^{295}$ with histidine residue. (B) AblPP can be quickly and tightly induced. The host HEK293-AblPP cells were treated with 2 µg/ml Doxy for different lengths of time. The cells were then collected, whole cell lysates were subjected to SDS-PAGE separation, and AblPP protein was detected with an anti-HA antibody; the whole cell lysates were also immunoblotted with antiphosphotyrosine antibody 4G10 in a different gel. (C) AblPP induction causes host HEK293-AblPP cells to detach from the supporting matrix. HEK293-AblPP cells were seeded on coverslips and cultured overnight. AblPP protein was induced by Doxy for 2 h in the presence or absence of 5 µg/ml imatinib. The actin was stained with FITC-phalloidin. Bars, 25 µm. (D) Blocking Abl kinase activity prevents the detachment of HEK293-AblPP cells. HEK293-AblPP cells were treated with Doxy for different lengths of time in the absence or presence of 5 µg/ml imatinib. Detached cells were counted as described in Materials and methods. The histogram shows normalized mean values ± SEM from three independent experiments. (E) The inducible expression of AblPP and AblKD. Cells were treated with Doxy for 5 h. The induced AblPP and AblKD proteins were detected using an anti-HA antibody. (F) Abl kinase activity is required for detachment of HEK293-AblPP cells. Cells were treated with Doxy for 2 h. The detached cells were counted as described in Materials and methods. The histogram shows normalized mean values ± SEM from three independent experiments.

(Jin and Wang, 2007). Collectively, the current results suggest that the Abl–CrkII signaling module can target different downstream effectors to exert a negative effect on cell spreading and cell migration.

The affinity and avidity of integrin receptors are regulated by a variety of factors in the process known as inside-out signaling (Hynes, 1987, 2002; Ginsberg et al., 2005). The small GTPase Rap1 is an important transducer of inside-out signals to activate integrins (Bos et al., 1997, 2001). One of the GEFs for Rap1 is C3G, which contains several proline-rich sequences that associate with the N-terminal SH3 domain of CrkII (Gotoh et al., 1995). Similar to DOCK180, C3G can be recruited to the plasma membrane through CrkII and p130Cas to stimulate Rap1-GTP (Ichiba et al., 1999). We show here that Abl-dependent tyrosine phosphorylation of CrkII causes the down-regulation of Rap1-GTP and a decrease in the affinity of β1 integrin. Interestingly, the Abl-mediated reduction in Rap1-GTP and integrin affinity is not sufficient to cause cell detachment. We have found that the Rho–ROCK1 pathway, activated by serum factors such as lysophosphatidic acid (LPA), is also required for cells to detach from the supporting matrix. Furthermore, we show that ephrin-A1–induced retraction of prostate cancer PC3 cells is dependent on the Abl–CrkII and Rho–ROCK pathways.

## Results

### Activated Abl kinase induces cell detachment

We adopted the T-Rex system to express the activated AblPP protein under the control of a Tet-on promoter in HEK293 cells (Hillen et al., 1983). The AblPP protein contains two substitutions mutations, P242E and P249E (Barila and Superti-Furga, 1998), which disrupt the interaction between the Abl SH3 domain and its SH2 kinase linker (Nagar et al., 2003) and thus lead to the constitutive activation of the Abl kinase (Hantschel and Superti-Furga, 2004). We also expressed a kinase-defective Abl (K290H; AblKD) from the Tet-on promoter (Fig. 1 A; Welch and Wang, 1995). The expression of AblPP or AblKD was induced with doxycycline (Doxy; Fig. 1, B and E). As expected, the induced expression of AblPP led to an increase in the tyrosine phosphorylation of cellular proteins (Fig. 1 B). Within 2 h of AblPP induction, the majority of cells (~80%) adopted a rounded morphology and detached from the dish (Fig. 1, C and D). The cell detachment response to Doxy was blocked by imatinib, a small molecule inhibitor of the Abl kinase (Fig. 1, C and D). Furthermore, Doxy did not induce the detachment of HEK293-AblKD cells (Fig. 1, E and F), nor did it induce the detachment of cells expressing exogenous Abl, which is autoinhibited

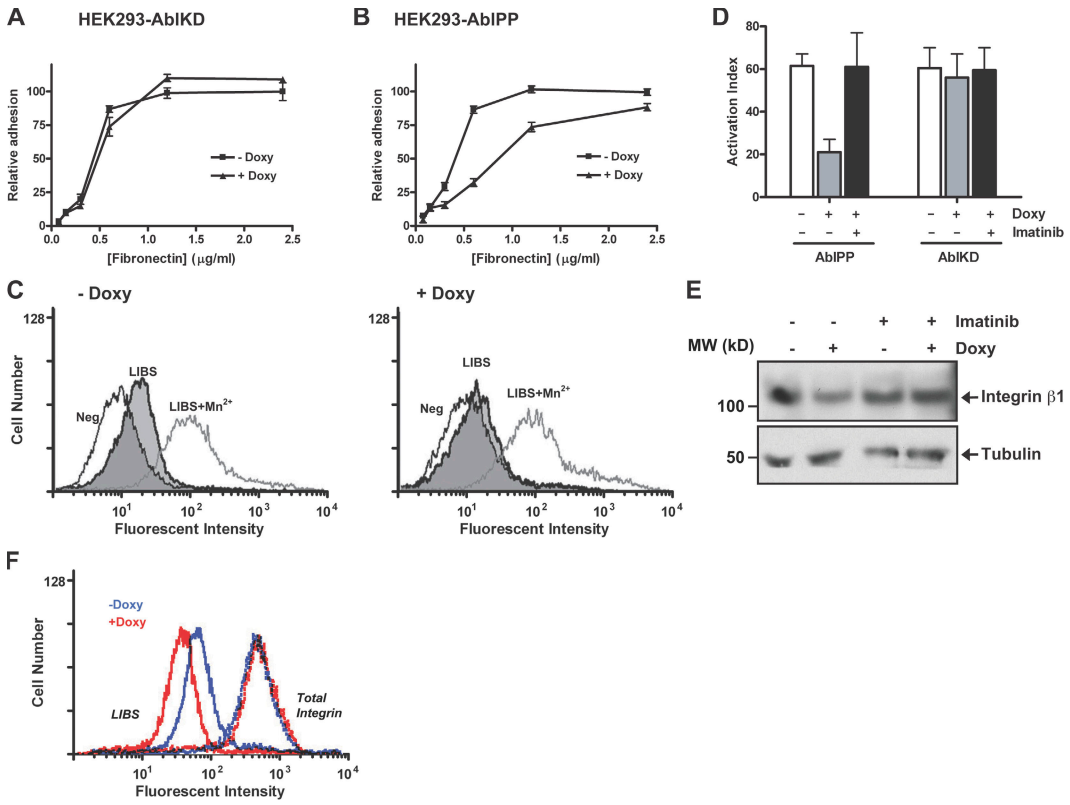


Figure 2. **Abl kinase–dependent reduction of β1 integrin affinity for fibronectin.** (A and B) The active Abl kinase caused a decrease in cell adhesion. Cells were treated with Doxy for 2 h. Cell adhesion was measured as described in Materials and methods. The data were from three technical repeats. The mean cell adhesion activity under 2.5 μg/ml fibronectin was set as 100. (C) Induction of AblPP suppresses integrin activation. HEK293-AblPP cells were stained with monoclonal antibody B44, a reporter of β1 integrin activation (gray histogram), after treatment with (right) or without (left) Doxy to induce AblPP expression. Negative controls were stained with secondary antibody alone (neg, black outline histogram). To ensure that integrins were not generally deficient in their capacity to activate, 200 μM $Mn^{2+}$ was added to externally activate cell surface integrins; these positive controls were also stained with B44 antibody (gray outlined histogram). (D) The mean level of β1 integrin activation is lower after AblPP induction in HEK293-AblPP cells, but not after AblKD induction in HEK293-AblKD cells. The histogram shows normalized mean values ± SEM (quantified from FACS) from three independent experiments. The integrin affinity measurement was performed as described in Materials and methods. (E) Induction of AblPP protein did not change the expression level of β1 integrins. HEK293-AblPP cells were treated with Doxy for 2 h, in the absence or presence of 5 μg/ml imatinib. β1 integrin levels were measured with anti–β1 integrin antibody P4C10 by using a nonreducing gel; anti-tubulin blot was used as loading control. (F) Induction of AblPP did not change total levels of β1 integrins. HEK293-AblPP cells were stained with monoclonal antibody B44 (solid histograms) or P4C10 (dotted histograms), in the absence (blue) or presence (red) of Doxy.

(Nagar et al., 2003) and unable to increase protein tyrosine phosphorylation in HEK293 cells (not depicted). These results suggest that the induced expression of an activated Abl kinase acutely interferes with cell adhesion.

To determine the effect of AblPP on cell adhesion, we measured the number of adherent cells as a function of fibronectin concentration before or after Doxy treatment of HEK293-AblKD and HEK293-AblPP cells (Fig. 2, A and B). The expression of AblKD did not affect cell adhesion to fibronectin (Fig. 2 A). In contrast, the expression of AblPP shifted the fibronectin dose–response curve to the right, indicating reduced adhesion in cells expressing an activated Abl kinase (Fig. 2 B). The HEK293 cells express β1 integrin, which is a receptor for fibronectin (Hynes, 2002). Consistent with reduced adhesion to fibronectin, we found that induction of AblPP caused a reduction in the affinity of β1 integrin (Fig. 2, C, D, and F), measured by reactivity to a monoclonal antibody B44, which detects an epitope, named ligand-induced binding site, present on active β1 integrin (Wilkins et al., 1996). This reduction in β1 integrin affinity was blocked by treatment of Doxy-induced HEK293-AblPP cells with imatinib (Fig. 2 D). Under the same condition, Doxy treatment did not affect the overall levels of β1 integrin on the cell surface or in whole cell lysates; in addition, Doxy treatment did not affect the number of β1 integrins that could be artificially activated by $Mn^{2+}$ (Fig. 2, C, E, and F). Furthermore, Doxy induction of AblKD expression did not influence the reactivity of β1 integrin with B44 antibody (Fig. 2 D). Collectively, these results show that the AblPP-induced cell detachment is associated with a decrease in the affinity of β1 integrin.

### Down-regulation of Rap1-GTP through Abl kinase–dependent disruption of the CrkII–C3G complex

Previous studies have documented the small GTPase Rap1 to play a critical role in the inside-out activation of integrins (Caron et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000). Because AblPP expression led to a reduction in the affinity of β1 integrin, we examined whether AblPP affected the levels of Rap1-GTP. We observed a consistent threefold reduction in the levels of Rap1-GTP within 2 h after treatment of HEK293-AblPP

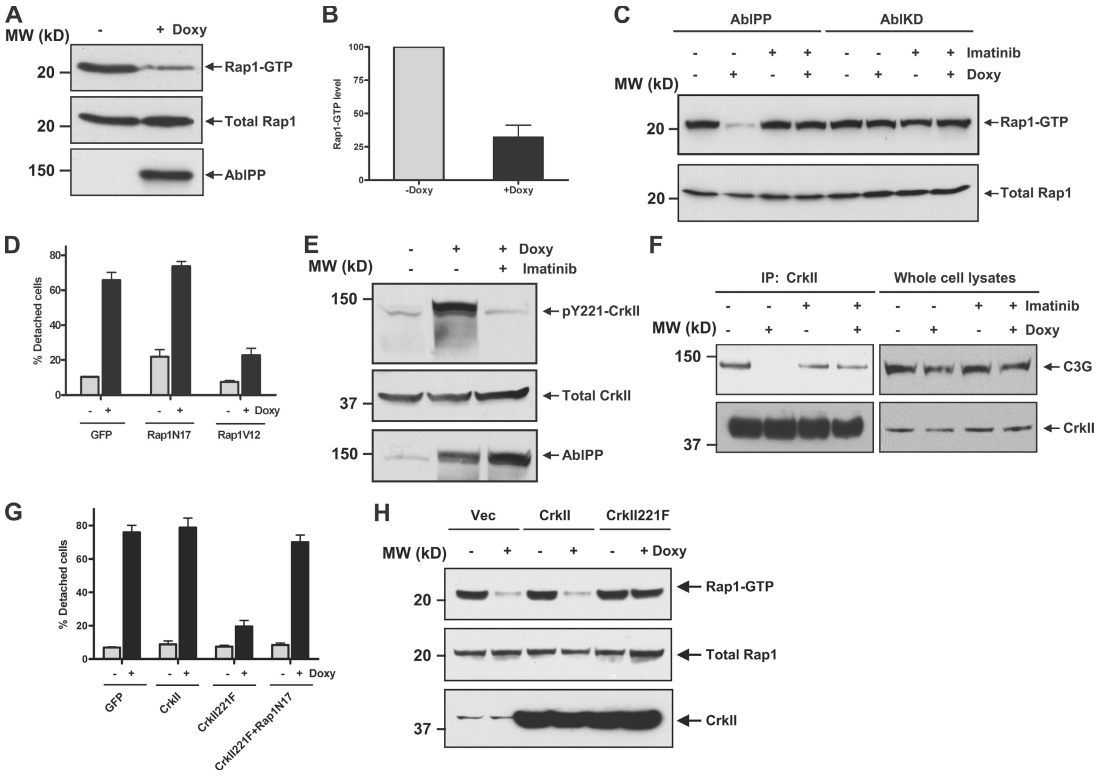


Figure 3. **Abl kinase–dependent down-regulation of Rap1 through CrkII–C3G.** (A) The active Abl kinase caused a decrease in the levels of Rap1-GTP. HEK293-AblPP cells were treated with Doxy for 2 h. Rap1-GTP levels were measured as described in Materials and methods. (B) The mean level of Rap1-GTP dropped after AblPP induction. The histogram shows normalized mean values ± SEM (quantified from immunoblots) from three independent experiments. (C) Abl kinase activity is required for down-regulation of Rap1-GTP levels. Cells were treated with Doxy for 2 h in the absence or presence of imatinib. Rap1-GTP levels were measured as described in Materials and methods. (D) A constitutively active Rap1 mutant (Rap1V12) abrogated AblPP-induced cell detachment. HEK293-AblPP cells were transfected with 2 µg GFP, Rap1N17, or Rap1V12 plasmids for 48 h. The cells were then treated with Doxy for 2 h. Detached cells were counted as described in Materials and methods. (E) Active Abl kinase phosphorylates CrkII on Tyr221. HEK293-AblPP cells were treated with Doxy for 2 h. The CrkII immunoprecipitates were probed with an antibody recognizing phosphorylated Tyr221 of CrkII and then reprobed with an antibody to CrkII. (F) CrkII forms a complex with C3G and this complex was disrupted by AblPP induction. HEK293-AblPP cells were treated with Doxy for 2 h. Western blots of CrkII immunoprecipitates were probed with anti-CrkII or anti-C3G antibodies. Whole cell lysates were probed with the same antibodies to be used as loading controls. (G) CrkII-221F abrogated AblPP-induced detachment of HEK293-AblPP cells. HEK293-AblPP cells were transfected with 2 µg CrkII, CrkII-221F, or CrkII-221F plus Rap1N17 plasmids for 48 h. The cells were then subjected to treatment with Doxy for 2 h. Detached cells were counted as described in Materials and methods. The histogram shows normalized mean values ± SEM from three independent experiments. (H) CrkII-221F abrogated AblPP-induced decrease of Rap1-GTP levels. HEK293-AblPP cells were transfected with 2 µg of vector, CrkII, or CrkII-221F plasmids for 48 h. The cells were then subjected to Doxy treatment for 2 h. Rap1-GTP levels were measured as described in Materials and methods.

cells with Doxy (Fig. 3, A and B). Imatinib treatment abrogated the reduction in Rap1-GTP levels induced by Doxy in AblPP-expressing cells (Fig. 3 C). Furthermore, the reduction in Rap1-GTP levels did not occur upon Doxy induction of AblKD expression (Fig. 3 C). Ectopic expression of the constitutively active Rap1V12 largely blocked cell detachment caused by AblPP, whereas the dominant-negative Rap1N17 did not cause cell detachment without AblPP (Fig. 3 D). These results suggest that the down-regulation of Rap1-GTP by AblPP is necessary but may not be sufficient to cause cell detachment.

One of the GEFs for Rap1 is C3G (Gotoh et al., 1995). The C3G protein binds to the N-terminal SH3 domain of the CrkII adaptor protein (Feller et al., 1994; Matsuda et al., 1994; Ren et al., 1994; Tanaka et al., 1994); and formation of the CrkII–C3G complex leads to the stimulation of Rap1-GTP (Ohba et al., 2001). Because CrkII is a substrate of the Abl kinase (Feller, 2001), we tested whether AblPP could disrupt the CrkII–C3G complex. Upon induction of AblPP, phosphorylation of the endogenous CrkII protein at tyrosine-221 was significantly increased, and treatment with imatinib abrogated this enhanced CrkII phosphorylation (Fig. 3 E). As expected, C3G coimmunoprecipitated with CrkII in HEK293-AblPP cells before Doxy treatment (Fig. 3 F). After Doxy induction, C3G was no longer detected in the anti-CrkII immune complex (Fig. 3 F). The dissociation of C3G from CrkII was blocked by imatinib, showing the requirement of Abl kinase activity for the inhibition of C3G–CrkII interaction (Fig. 3 F). To further demonstrate that tyrosine phosphorylation of CrkII was responsible for AblPP-induced down-regulation of Rap1-GTP, we expressed the CrkII-221F mutant in HEK293-AblPP cells. We found that the expression of CrkII-221F abolished AblPP-induced cell detachment (Fig. 3 G) and that CrkII-221F expression prevented the loss of Rap1-GTP after AblPP induction (Fig. 3 H). The coexpression with Rap1N17 abrogated the effect of CrkII-221F and restored AblPP-induced cell detachment (Fig. 3 G). Thus, AblPP-dependent phosphorylation of CrkII on Y221 causes the disruption of the CrkII–C3G complex, the reduction in the levels of Rap1-GTP, and cell detachment.

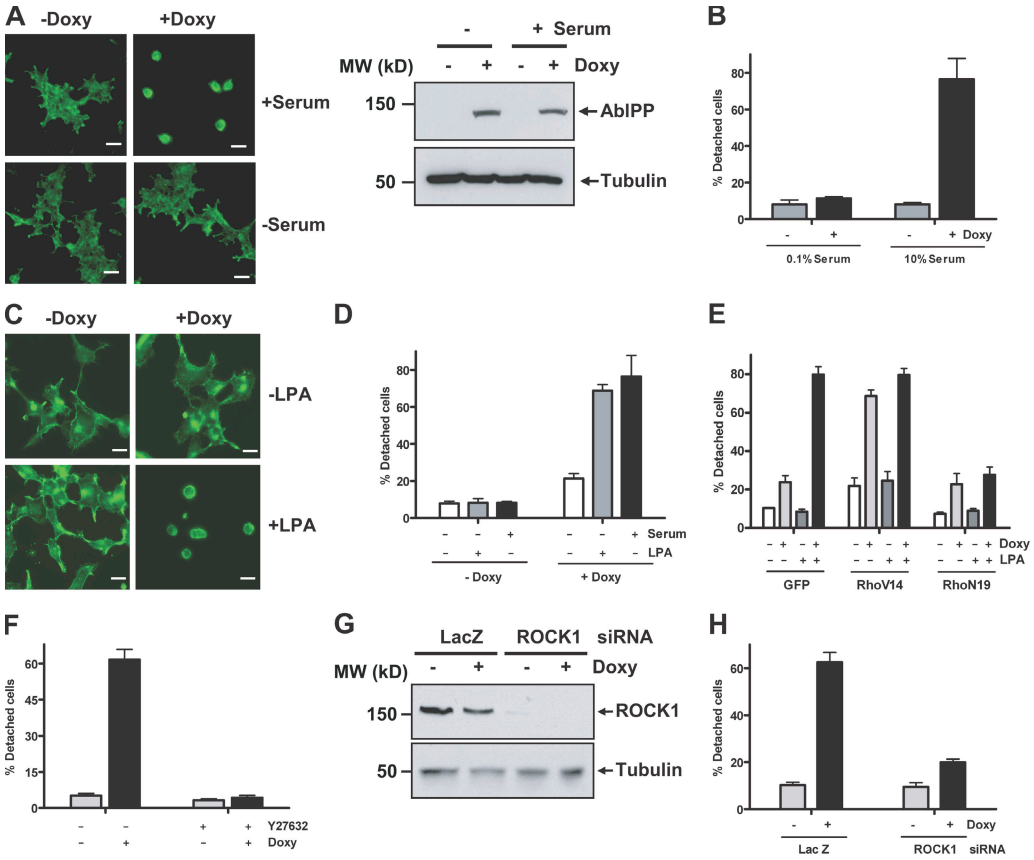


Figure 4. **Requirement for the Rho–ROCK1 pathway in cell detachment.** (A, left) Expression of AblPP induced the HEK293-AblPP cells to detach from supporting matrix in complete media but not in serum-free media. HEK293-AblPP cells were cultured overnight; the media were then changed to fresh DME supplemented with 10 or 0.1% of serum. The cells were then treated with Doxy for 2 h. Cells were fixed and actin was stained with FITC-phalloidin. Bars, 25 μm. (right) The whole cell lysates from different conditions were subjected to SDS-PAGE separation and AblPP protein was detected with an anti-HA antibody. (B) The mean level of cell detachment of HEK293-AblPP cells in DME, supplemented with 10 or 0.1% of serum. The histogram shows normalized mean values ± SEM from three independent experiments. (C) Activated Abl and LPA were both required to induce the host HEK293-AblPP cells to detach. HEK293-AblPP cells were treated with Doxy for 2 h in serum-free media, in the presence or absence of 1 μg/ml LPA. Cells were then fixed and actin was stained with FITC-phalloidin. Bars, 25 μm. (D) The mean level of cell detachment of HEK293-AblPP cells in fresh DME, treated with or without Doxy, in the presence or absence of LPA. The histogram shows normalized mean values ± SEM from three independent experiments. (E) Rho activity is required for activated Abl to induce cells to detach. HEK293-AblPP cells were transfected with the constitutively activated RhoV14 or the dominant-negative RhoN19 constructs for 48 h. Cells were then treated with Doxy for 2 h. The detached cells were counted as described in Materials and methods. The histogram shows normalized mean values ± SEM from three independent experiments. (F) A specific inhibitor of ROCK1, Y27632, can completely block AblPP-induced HEK293-AblPP cell detachment. HEK293-AblPP cells were pretreated with or without 20 nM Y27632 for 1 h and then treated with Doxy for 2 h. The detached cells were counted as described in Materials and methods. (G) Knockdown of ROCK1. HEK293-AblPP cells were transfected with control siRNA *(LacZ)* or *ROCK1* siRNA. 48 h after transfection, both pools of the cells were treated with or without Doxy for 2 h. Whole cell lysates were then collected and probed with anti-ROCK1 antibodies; anti-tubulin blot was used as a loading control. (H) Knockdown of ROCK1 inhibited AblPP-induced HEK293-AblPP cell detachment. 48 h after transfection with the indicated siRNA, HEK293-AblPP cells were treated with Doxy for 2 h. The detached cells were counted as described in Materials and methods. The histogram shows normalized mean values ± SEM from three independent experiments.

### Requirement of Rho and ROCK1 in cell detachment

During the course of these studies, we noticed that Doxy treatment did not cause cell detachment in serum-free media (Fig. 4, A and B), despite similar levels of induction of the AblPP protein (Fig. 4 A). We postulated that cell detachment might also require contractility that is stimulated by serum factors. It is well established that actin-myosin–based cellular contractility is stimulated by the Rho-GTP–activated protein kinase ROCK1 (Kimura et al., 1996, 1998) and that the Rho–ROCK1 pathway is activated by mitogenic factors such as LPA (Moolenaar, 1995). LPA binds a G protein–coupled receptor to activate heterotrimeric G proteins ($G_{\alpha 12/13}$), leading to the activation of the Rho–ROCK1 pathway (Hart et al., 1998; Kozasa et al., 1998). Consistent with our hypothesis, we found that neither LPA nor Doxy alone induced rounding and detachment of the HEK293-AblPP cells, but the combined treatment (LPA plus Doxy) caused cell rounding and detachment in serum-free media (Fig. 4, C and D).

To further demonstrate the requirement of Rho, we expressed a constitutively active RhoV14 or a dominant-negative RhoN19 in HEK293-AblPP cells and monitored cell detachment after AblPP induction. Cells expressing RhoV14 underwent detachment after AblPP induction in the absence of LPA or serum (Fig. 4 E), showing that constitutively active Rho could supplant the LPA or serum requirement. Furthermore, the dominant-negative RhoN19 largely, if not completely, abrogated cell detachment even when AblPP was induced in the presence of LPA or serum (Fig. 4 E). An important downstream effector of Rho

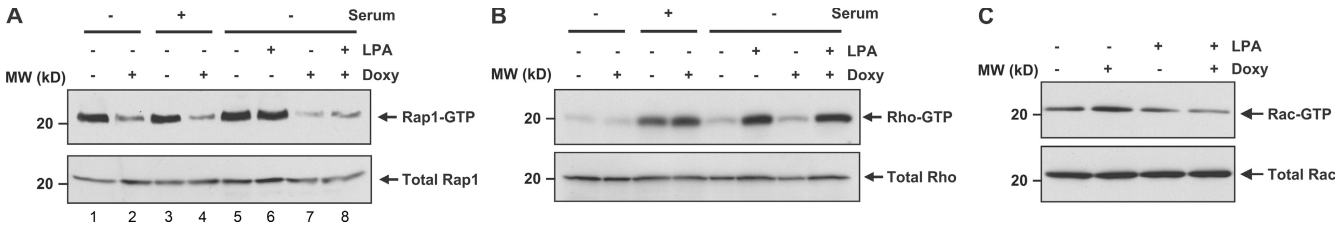


Figure 5. **Down-regulation of Rap1-GTP by activated Abl kinase is independent of Rho-GTP up-regulation by LPA.** (A and B) HEK293-AblPP cells were cultured overnight and were kept in complete media (+ serum) or changed to fresh DME (− serum) for 2 h. The cells were then treated with or without Doxy for 2 h. The cells in DME (lanes 5–8) were also subject to LPA treatment (lanes 6 and 8) for 5 min, in the presence (lanes 7 and 8) or absence (lanes 5 and 6) of AblPP by treating with Doxy for 2 h. Rap1-GTP (A) and Rho-GTP (B) were then pulled down from whole cell lysates as described in Materials and methods. Whole cell lysates were also resolved and immunoblotted with antibodies to Rap1 or Rho as loading controls. (C) HEK293-AblPP cells were cultured overnight and were then changed to fresh DME for 2 h. The cells were then treated with or without Doxy for 2 h. After that, cells were treated with or without LPA for 5 min. Rac-GTP was pulled down from whole cell lysates as described in Materials and methods. Whole cell lysates were also resolved and immunoblotted with antibody to Rac as loading controls.

in contractility is the ROCK1 kinase (Kimura et al., 1996, 1998), which can be inhibited by the compound Y27362 (Fujisawa et al., 1996). We found that treatment of HEK293-AblPP cells with Y27362 completely blocked the Doxy-induced cell detachment (Fig. 4 F). We also knocked down ROCK1 in HEK293-AblPP cells with siRNA and found that the reduction in ROCK1 significantly lowered the percentage of detached cells after AblPP induction (Fig. 4, G and H). These data provide further evidence that the Rho–ROCK1 pathway is required for AblPP-induced cell detachment.

### Inhibition of Rap1 and activation of Rho are independent pathways in cell detachment

Results shown in Figs. 3 and 4 suggest down-regulation of Rap1-GTP and up-regulation of Rho-GTP are both required for cell detachment after the induced expression of AblPP. We therefore tested whether AblPP could directly affect the activity of Rho and whether LPA could directly affect the activity of Rap1. As shown in Fig. 5 A, Doxy induction of AblPP caused similar reductions in the Rap1-GTP levels in the absence or presence of serum or LPA. In contrast, LPA did not affect the levels of Rap1-GTP when AblPP was not induced. These results show that serum factors are dispensable for the inhibition of Rap1 by AblPP. In contrast, induced expression of AblPP did not increase the levels of Rho-GTP, either in the presence or the absence of serum. The activation of Rho-GTP by LPA was similarly unaffected by Doxy induction of AblPP (Fig. 5 B). These results show that AblPP does not influence Rho-GTP levels. Given the previous findings that CrkII phosphorylation is associated with the down-regulation of Rac-GTP (Kain and Klemke, 2001), we also measured the levels of Rac-GTP and found that neither AblPP induction nor LPA reduced the levels of Rac-GTP in this experimental system (Fig. 5 C). Collectively, these data show that down-regulation of Rap1-GTP by activated Abl kinase and up-regulation of Rho-GTP by LPA are independent pathways, and both are required to cause cell detachment.

### Requirement of Abl kinase and LPA in ephrin-induced cell rounding

The aforementioned experiments with the HEK293-AblPP cells have led to the conclusion that acute activation of Abl kinase can cause cell rounding and detachment in conjunction with the Rho–ROCK1 pathway. A previous paper has shown that stimulation of PC3 prostate cancer cells with ephrin-A1 reduces their adhesion to fibronectin (Miao et al., 2000). Ephrin-A1 is shown to activate the EphA2 receptor tyrosine kinase in PC3 cells (Miao et al., 2000). Previous studies have also shown that Abl is a downstream effector of activated Eph family of tyrosine kinase receptors and that stimulation of EphB4 receptor in breast cancer cells leads to Abl-dependent tyrosine phosphorylation of CrkII at Y221 (Harbott and Nobes, 2005; Pasquale, 2005, 2008; Noren et al., 2006). We therefore tested if Abl kinase plays a role in ephrin-A1–induced detachment of PC3 cells.

Stimulation of PC3 cells with Fc-conjugated ephrin-A1 induced a rapid and transient tyrosine phosphorylation of the endogenous Abl protein, peaking at 10 min and decaying by 30 min (Fig. 6, A and B). Imatinib reduced the tyrosine phosphorylation of the Abl protein, indicating autophosphorylation (Fig. 6 A). The addition of Fc-ephrin-A1 also induced a rapid and transient retraction of PC3 cells, in keeping with previous results (Miao et al., 2000; Fig. 6, C and D). Interestingly, imatinib reduced this retraction response by about threefold (Fig. 6, C and D), suggesting a role for Abl kinase in Fc-ephrin-A1–induced cell rounding and detachment. Similar to the effect of AblPP in HEK293 cells, activation of the endogenous Abl kinase by Fc-ephrin-A1 caused a reduction in the levels of Rap1-GTP, and this effect was blocked by imatinib (Fig. 6 E). To further demonstrate the requirement of Abl, we stably expressed a lentiviral Abl small hairpin RNA (shRNA) or control shRNA in PC3 cells (Fig. 6 F). Treatment with Fc-ephrin-A1 reduced the B44 reactivity of β1 integrin in PC3 cells, and this reduction was abolished in Abl knocked down PC3 cells (Fig. 6 G). In PC3 cells, stimulation with Fc-ephrin-A1 disrupted the C3G coimmunoprecipitation with CrkII (Fig. 6 H, left panels). The disruption of the C3G–CrkII complex was not observed in Fc-ephrin-A1–stimulated PC3 cells transduced with the Abl-shRNA (Fig. 6 H, right panels). These results show that Fc-ephrin-A1 activates Abl tyrosine kinase in PC3 cells and that Abl is required for the reduced adhesion, the down-regulation of Rap1-GTP, and the disruption of the CrkII–C3G complex.

We also tested the requirement of Rho and ROCK1 in Fc-ephrin-A1–induced PC3 cell retraction. It has been reported that activated Eph receptors can stimulate Rho-GTP (Shamah et al., 2001; Sahin et al., 2005) and that the Rho–ROCK1

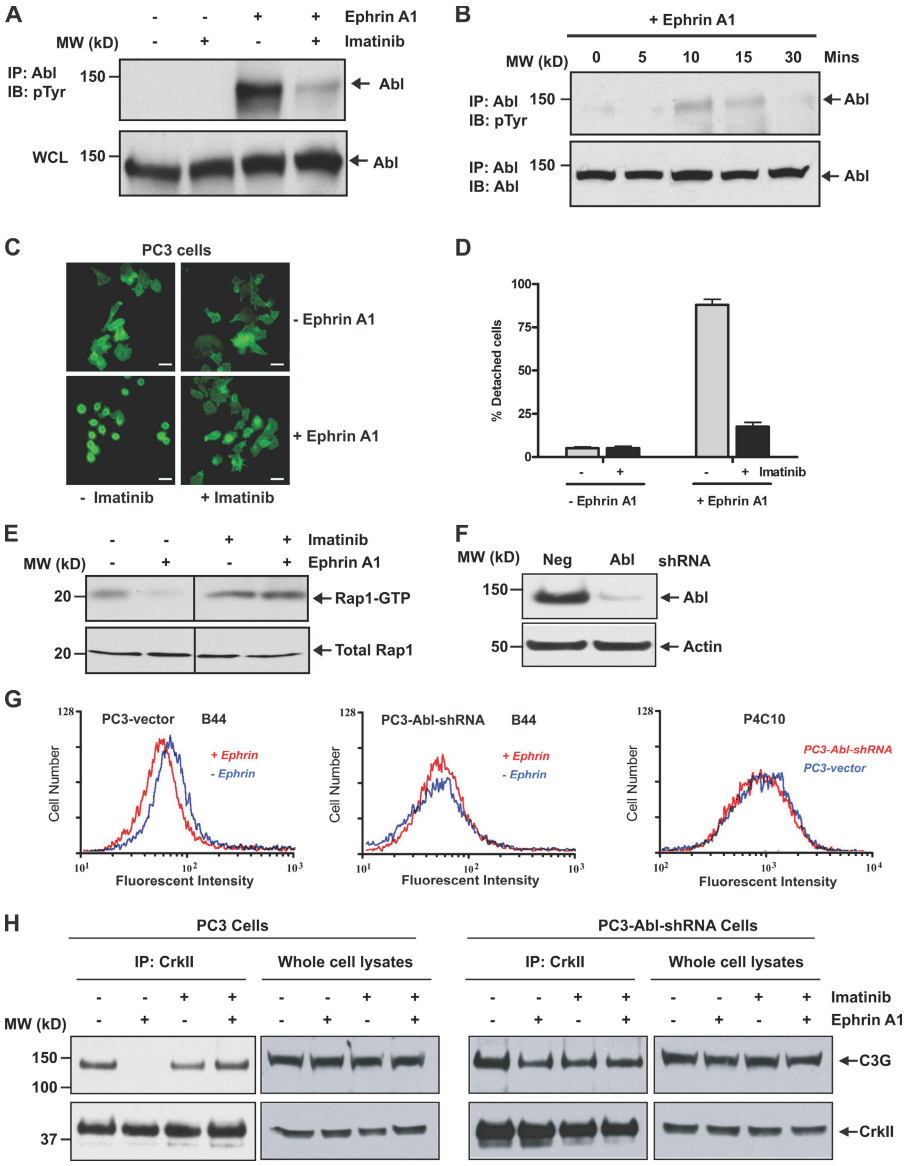


Figure 6. **Abl–Rap1 pathway is required in ephrin-A1–induced PC3 cell detachment.** (A) Endogenous c-Abl was phosphorylated within 15 min after treatment of PC3 cells with Fc-ephrin-A1. After 15 min of treatment, either in the absence or presence of 5 μg/ml imatinib, PC3 cells were collected. Western blots of Abl immunoprecipitates were probed with the anti-phosphotyrosine antibody 4G10. Whole cell lysates were probed with anti-Abl antibody as loading control. (B) Time course experiment of c-Abl phosphorylation after treatment of PC3 cells with Fc-ephrin-A1. PC3 cells were treated with Fc-ephrin-A1 for different lengths of time. Western blots of Abl immunoprecipitates were probed with the antiphosphotyrosine antibody 4G10. (C) Imatinib partially blocked ephrin-A1–induced PC3 cell detachment. PC3 cells were treated with Fc-ephrin-A1 for 10 min, in the absence or presence of imatinib. Cells were then fixed and actin was stained with FITC-phalloidin. Bars, 25 μm. (D) The mean level of detached PC3 cells was reduced by pretreatment with imatinib in PC3 cells. The histogram shows normalized mean values ± SEM from three independent experiments. (E) Ephrin-A1 treatment led to a reduction in Rap1-GTP levels, but could be rescued by pretreatment with imatinib. PC3 cells were subjected to Fc-ephrin-A1 treatment for 10 min, and Rap1-GTP was pulled down and detected as described in Materials and methods. (F) Generation of PC3-Abl-shRNA cells. PC3 cells were infected by lentiviral shRNA construct or the control vector. Whole cell lysates from the selected cells were subjected to SDS-PAGE separation and Abl protein was detected with an anti-Abl antibody. (G) Knockdown of endogenous c-Abl in PC3 cells prevents β1 integrin affinity decrease after ephrin-A1 treatment. PC3 and the PC3-Abl-shRNA cells were subjected to Fc-ephrin-A1 treatment for 10 min (left and middle, red) and compared with untreated controls (left and middle, blue) for staining with the active integrin-specific antibody B44. Although ephrin-A1 treatment selectively decreased integrin activation in cells expressing c-Abl, the overall level of integrin was the same in cells regardless of c-Abl expression (right, red vs. blue). (H) Knockdown of Abl in PC3 cells prevents CrkII–C3G complex dissociation after ephrin-A1 treatment. PC3 cells and PC3-Abl-shRNA cells were treated with Fc-ephrin-A1 for 10 min, in the absence or presence of imatinib. Western blots of CrkII immunoprecipitates were probed with anti-CrkII or anti-C3G antibodies. Whole cell lysates were probed with the same antibodies as loading controls.

pathway is required for Eph-induced cell retraction (Wahl et al., 2000; Lawrenson et al., 2002). Consistently, we found that the ROCK1 kinase inhibitor (Y27632) completely blocked the rounding response to Fc-ephrin-A1 in PC3 cells (Fig. 7, A and B). However, we also found that Fc-ephrin-A1–induced cell rounding was significantly reduced in serum-free media and it could be restored by the addition of LPA (Fig. 7, C and D). We compared the effects of Fc-ephrin-A1 and LPA on the levels of Rho-GTP in serum-starved PC3 cells and found that Fc-ephrin-A1 caused a transient activation of Rho-GTP at a level that was much lower than that stimulated by LPA (Fig. 7 E). Collectively, results in Fig. 7 (A–E) suggest that Eph-dependent activation of Rho is not sufficient and that the additional simulation of the Rho–ROCK1 pathway by LPA is also necessary to induce cell retraction. To rule out the possibility that high concentrations of Fc-ephrin-A1 might have caused receptor down-regulation and thus limited the levels of Rho-GTP, we tested lower concentrations and observed a similar requirement for LPA in cell detachment irrespective of Fc-ephrin-A1 concentrations (Fig. 7 F). Thus, in PC3 cells, Fc-ephrin-A1 alone was sufficient to activate Abl kinase and to reduce Rap1-GTP but not sufficient to activate the Rho–ROCK1 pathway to a level that can induce cell rounding. To further demonstrate the separation of the Abl–Rap1 and the Rho–ROCK1 pathways, we show that the ROCK1 inhibitor does not block Fc-ephrin-A1–induced disruption of the CrkII–C3G complex (Fig. 7 G). Therefore, the two independent pathways shown to be required for the detachment of HEK293-AblPP cells were also required for the Fc-ephrin-A1–induced retraction of PC3 cells (Fig. 8).

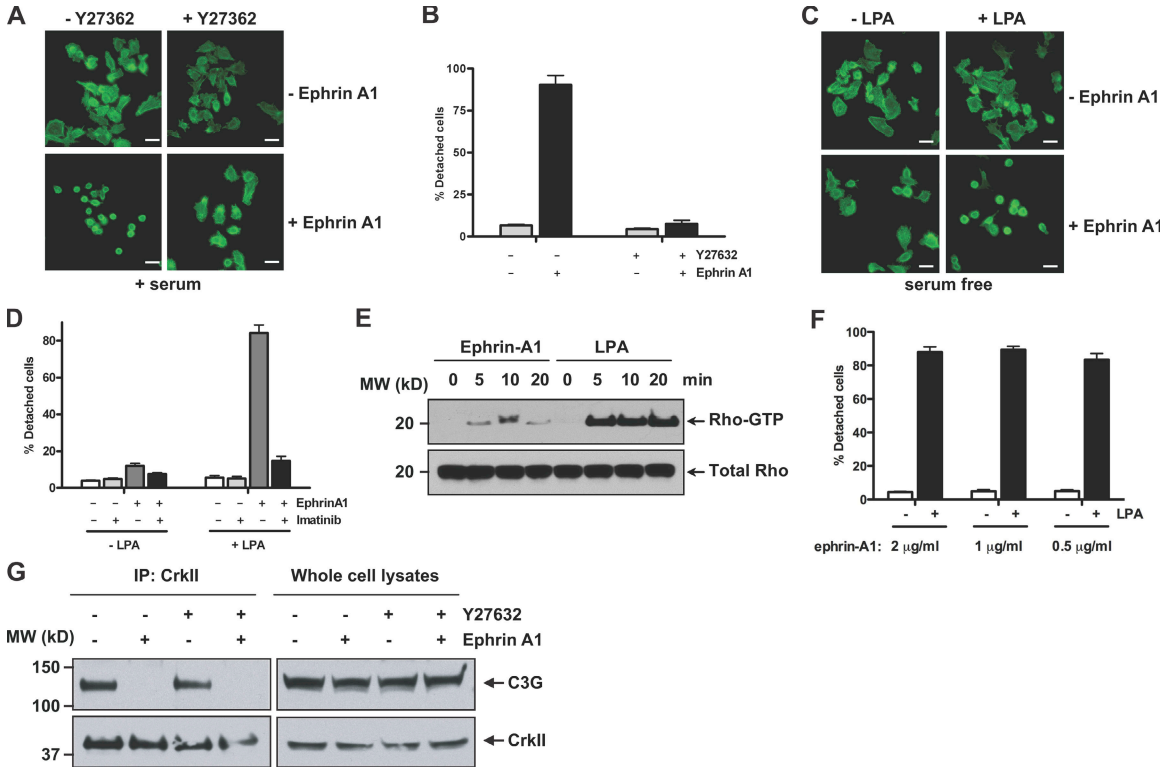


Figure 7. **Rho–ROCK1 pathway is also required in ephrin-A1-induced PC3 cell detachment.** (A) ROCK1 activity was required for ephrin-A1–induced PC3 cell detachment. PC3 cells were treated with Fc-ephrin-A1 for 10 min, either in the absence or presence of 20 nM Y27632. Cells were then fixed and actin was stained with FITC-phalloidin. Bars, 25 μm. (B) Y27632 blocked ephrin-A1–induced PC3 cell detachment. The histogram shows normalized mean values ± SEM from three independent experiments. (C) LPA restored PC3 cell detachment induced by ephrin-A1 in serum-free media. PC3 cells were cultured overnight in 0.1% serum and changed to fresh media without serum for 4 h. Cells were then treated with Fc-ephrin-A1 for 15 min, in the absence or presence of 1 μg/ml LPA. Cells were then fixed and actin was stained with FITC-phalloidin. Bars, 25 μm. (D) The mean level of cell detachment of PC3 cells in serum-free media after Fc-ephrin-A1 treatment, in the presence or absence of LPA. The histogram shows normalized mean values ± SEM from three independent experiments. (E) Activation of Rho by ephrin-A1 or LPA. PC3 cells were cultured overnight in 0.1% serum and changed to fresh media without serum for 4 h. Cells were then treated with Fc-ephrin-A1 or LPA. At the indicated time (in minutes), cells were harvested and Rho-GTP was measured as described in Materials and methods. (F) The mean level of cell detachment of PC3 cells after different concentrations of Fc-ephrin-A1 treatment. PC3 cells were cultured overnight in media with 0.1% serum and changed to fresh media without serum for 4 h and then treated with the indicated concentrations of Fc-ephrin-A1 for 15 min, in the absence or presence of 1 μg/ml LPA. Rounded cells were counted. The histogram shows normalized mean values ± SEM from three independent experiments. (G) ROCK1 inhibitor does not interfere with the CrkII–C3G complex dissociation after ephrin-A1 treatment. Exponentially growing PC3 cells were treated with Fc-ephrin-A1 for 10 min, in the absence or presence of 20 nM Y27632. Western blots of CrkII immunoprecipitates were probed with anti-CrkII or anti-C3G antibodies. Whole cell lysates were probed with the same antibodies as loading controls.

## Discussion

### Abl-dependent reduction of Rap1-GTP downstream of Eph

We show here that activation of Abl tyrosine kinase causes reduction in the levels of Rap1-GTP through CrkII tyrosine phosphorylation and the disruption of the CrkII–C3G complex (Figs. 3, 5, and 6). Consistent with the function of Rap1-GTP as an activator of integrins (Reedquist et al., 2000; Bos et al., 2001; Bertoni et al., 2002), Abl-dependent reduction of Rap1-GTP leads to a reduction in the affinity of β1 integrin (Figs. 2 and 6). We further show that Fc-ephrin-A1 activates Abl kinase and causes Abl-dependent disruption of the CrkII–C3G complex and the down-regulation of Rap1-GTP in PC3 prostate cancer cells (Fig. 6). This Abl-dependent pathway is likely to contribute to the previously reported interference of cell adhesion by Fc-ephrin-A1 in PC3 cells (Miao et al., 2000).

Two recent reports have shown that activation of EphA4 by ephrin-A1 or activation of EphB2 by ephrin-B1 caused the reduction of Rap1-GTP in neurons or colon cancer cells, respectively (Riedl et al., 2005; Richter et al., 2007). In neurons, the ephrin-A1–induced down-regulation of Rap1-GTP involves SPAR, a Rap1-GAP that is recruited to the EphA4 receptor (Richter et al., 2007). The mechanism of Rap1-GTP down-regulation in colon cancer cells was not determined (Riedl et al., 2005). In breast cancer cells, it has been shown that ephrin-B2 activates Abl to phosphorylate CrkII at tyrosine-221 (Noren et al., 2006). Our results show that CrkII tyrosine phosphorylation contributes to the reduction of Rap1-GTP, via the dissociation of C3G from pY221–CrkII. Collectively, these results support the conclusion that reduction of Rap1-GTP is an important downstream effect of Eph family receptor activation and that this reduction can be achieved through the Abl–CrkII pathway described here as well as the direct recruitment of a Rap1-GAP to the activated receptor (Richter et al., 2007).

The adaptor protein CrkII forms a complex with C3G (Tanaka et al., 1994; Feller, 2001), which is a GEF for Rap1 (Reedquist et al., 2000). The SH2 domain of CrkII binds to

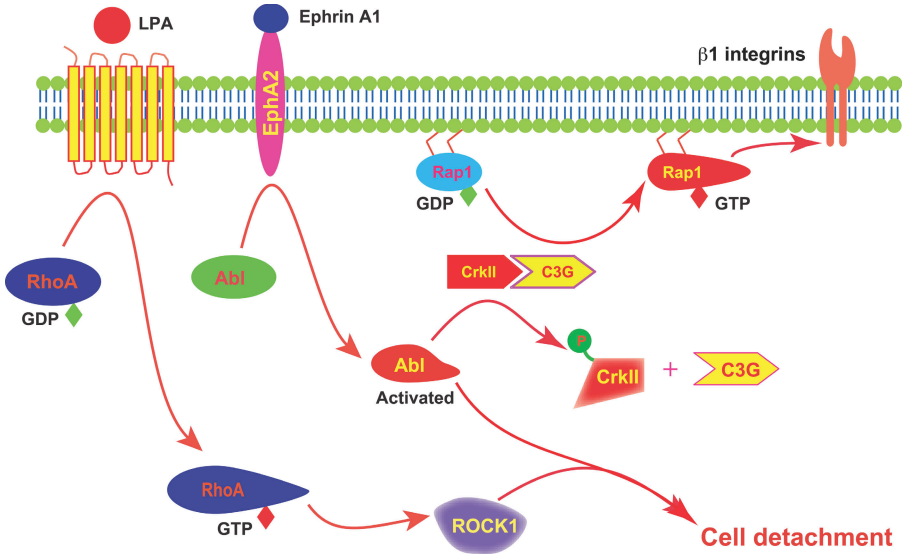


Figure 8. **Two parallel pathways are required for Abl-dependent cell detachment.** Activation of Abl kinase by ephrin-A1 disrupts the CrkII–C3G complex to lower the levels of Rap1-GTP and thus reduces the affinity of β1 integrin. In parallel, serum factors such as LPA strongly activate the Rho–ROCK1 pathway to stimulate cellular contractility. The combination of a reduced level of cell adhesion and a high level of cellular contractility causes cell detachment from the extracellular matrix.

tyrosine-phosphorylated p130Cas (Kain et al., 2003; Holcomb et al., 2006), allowing for the recruitment of CrkII–C3G to the plasma membrane and the activation of membrane-bound Rap1. Thus, tyrosine phosphorylation of p130Cas can lead to the activation of Rap1-GTP (Gotoh et al., 2000; Sakakibara et al., 2002). Previous studies have demonstrated that Y221 phosphorylation of CrkII induces an intramolecular interaction between pY221 and the SH2 domain of CrkII (Rosen et al., 1995), thus causing the dissociation of CrkII from pTyr-p130Cas (Kain and Klemke, 2001). However, this mechanism cannot explain the dissociation of C3G, which binds the CrkII SH3 domain (Knudsen et al., 1995; Wu et al., 1995). Previous studies have shown that pY221–CrkII does not form a complex with C3G (Okada et al., 1998; Feller, 2001), which is consistent with our results that Abl-dependent phosphorylation of CrkII at Y221 leads to the disruption of the C3G–CrkII complex. It thus appears that Y221 phosphorylation not only blocks the CrkII SH2 domain but also affects the ability of CrkII SH3 domain to bind proteins such as C3G (Feller, 2001). The mechanism underlying phosphorylation-mediated disruption of the CrkII–C3G complex is presently unclear. A possible clue to how pY221 may disrupt the CrkII–C3G complex is provided by previous findings that the pY221-SH2 intramolecular interaction can activate a latent SH3-binding site in the CrkII SH2 domain (Anafi et al., 1996; Donaldson et al., 2002). Perhaps this pY221-induced latent binding site may recruit another protein to cause the dissociation of C3G. With the overproduced AblPP protein, we have observed a kinase-dependent formation of the AblPP–CrkII complex upon Doxy induction of HEK293-AblPP cells (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200801192/DC1), correlating with the dissociation of C3G (Fig. 3 F). Because the latent SH3-binding site in the CrkII SH2 domain can bind to the Abl SH3 domain (Anafi et al., 1996; Donaldson et al., 2002), the overproduced AblPP protein itself appeared to bind to pY221–Crk and may thus contributing to the dissociation of C3G. However, we did not detect a stable CrkII–Abl complex in PC3 cells after Fc-ephrin-A1–induced activation of the endogenous Abl kinase (unpublished data). Therefore, we cannot attribute the dissociation of C3G from CrkII in PC3 cells to a direct competition by Abl, despite the fact that the dissociation of C3G is dependent on Abl in PC3 cells. It is conceivable that the pY221–CrkII protein, with the exposure of the latent SH3-binding site, may participate in an alternative protein–protein interaction to displace C3G and thus contribute to the reduction of Rap1-GTP.

### Cell detachment as a result of Abl activation requires Rho activity

Although Abl kinase reduces the affinity of β1 integrin, it is not sufficient to cause cells to detach from the supporting matrix (Fig. 4). We show that the activities of the small GTPase Rho and its downstream effector ROCK1 kinase are also required for cells to round up and detach (Fig. 4). Induced expression of AblPP does not activate the Rho–ROCK1 pathway in the absence of serum factors (Fig. 5), nor does it affect myosin light chain phosphorylation (not depicted). Instead, activation of Rho by serum factors such as LPA is required to collaborate with Abl kinase to cause cell detachment (Fig. 4). Because serum or LPA is not required for AblPP to reduce Rap1-GTP (Fig. 5), our results suggest that cell detachment occurs under conditions of Rap1-GTP down-regulation in combination with high levels of Rho-GTP (Fig. 8).

Interestingly, we show that serum or LPA also promotes Fc-ephrin-A1–induced rounding of PC3 prostate cancer cells (Fig. 7). Activation of the Eph family of receptors has been linked to the stimulation of Rho through several pathways in neurons and cancer cells (Wahl et al., 2000; Shamah et al., 2001; Lawrenson et al., 2002; Sahin et al., 2005; Parri et al., 2007; Fang et al., 2008). Those results are consistent with our finding that Fc-ephrin-A1 stimulation of PC3 cells caused a transient increase in Rho-GTP levels. However, we also show that the increase in Rho-GTP induced by Fc-ephrin-A1 in serum-free media is not sufficient to cause cell retraction. Instead, the additional and stronger activation of Rho-GTP by serum factors such as LPA is required for PC3 cells to retract. Collectively, the current literature and the results described here suggest that the limited stimulation of Rho-GTP by an activated Eph receptor may be sufficient

to cause localized retraction of axons during neuronal path finding, but not enough to induce the detachment of cancer cells. Our results also call for a reexamination of previous studies on the effects of Eph receptors, because the presence or absence of serum may have contributed to the varying biological effects that have been attributed to Eph receptor activation in cell culture–based experiments.

### Implications on the role of Abl kinase in cell migration and tumor metastasis

The Abl protein contains an F-actin binding domain (McWhirter and Wang, 1993; Hantschel et al., 2005), and the activated Abl kinase can stimulate actin polymerization (Woodring et al., 2003). Activation of Abl upon cell adhesion to fibronectin stimulates the formation of F-actin microspikes (Woodring et al., 2002, 2003) and the dorsal sequestration of Rac-GTP (Jin and Wang, 2007). Activation of Abl by growth factors stimulates the formation of membrane ruffles (Plattner et al., 1999; Sini et al., 2004), and activation of Abl by bacterial pathogens promotes their uptake, a process that requires the host cellular F-actin (Burton et al., 2003, 2005; Swimm et al., 2004). Although actin polymerization drives the forward movement of a migrating cell through lamellipodia extension at the front end, the existing data do not support a role for Abl in the stimulation of cell migration. On the contrary, Abl kinase inhibits cell migration stimulated by growth factors (Frasca et al., 2001; Kain and Klemke, 2001); it also inhibits cell spreading stimulated by fibronectin (Jin and Wang, 2007). In our previous study on the role of Abl during cell spreading, we found that the Abl kinase activity does not affect cell adhesion to fibronectin in fibroblasts (Jin and Wang, 2007). Consistent with those results, we found that the cellular Rap1-GTP levels were not affected during fibroblast spreading on fibronectin, either in the absence or the presence of Abl (Fig. S1 B). Instead, we have previously shown that Abl-dependent CrkII phosphorylation is involved in the dorsal sequestration of Rac-GTP during fibroblast spreading on fibronectin (Jin and Wang, 2007). Furthermore, although Kain and Klemke (2001) have shown Abl-dependent CrkII phosphorylation reduces the levels of Rac-GTP in transient transfection experiments, we did not observe any reduction in Rac-GTP levels after the induced expression of AblPP in HEK293 cells (Fig. 5 C). Together, results from this and previous studies suggest that the Abl–CrkII signaling module can exert different downstream effects on Rac-GTP or Rap1-GTP. The biological output from Abl–CrkII appears to be modulated by the upstream signals, i.e., growth factors versus ephrins versus fibronectin, as well as by the type of cells in which the Abl–CrkII module is activated.

Previous research has shown that the Abl–CrkII pathway is activated by Eph family of receptor tyrosine kinases, which regulate axon guidance, angiogenesis, and tissue patterning during embryonic development (Kullander and Klein, 2002; Pasquale, 2005; Pasquale, 2008). Recently, Eph receptors are also shown to be involved in tumor development, with either positive or negative effects on the tumor metastatic potential (Kinch et al., 2003; Miyazaki et al., 2003; Brantley-Sieders et al., 2004; Saito et al., 2004; Noren et al., 2006; Pasquale, 2008). The negative effect of EphB4 on breast cancer cell migration and invasion has been linked to the activation of Abl and the phosphorylation of CrkII (Noren et al., 2006). This conclusion is consistent with the inhibitory role of Abl in cell migration. However, other studies have found a positive correlation between the Abl kinase activity and the metastatic potential of breast cancer cells (Srinivasan and Plattner, 2006; Srinivasan et al., 2007). Metastasis is a complex process that requires tumor cells to escape from the tissue of origin, travel through the circulations, and colonize remote tissue sites (Chambers et al., 2000). Because activated Abl kinase inhibits cell migration (Woodring et al., 2003), it can interfere with tumor cell migration to distant sites; at the same time down-regulation of Rap1-GTP by activated Abl kinase may promote dissociation of tumor cells from the parent tissue. As a result, pharmacological inhibitors of the Abl kinase may block tumor cell escape from the tissue of origin but run the risk of promoting tumor migration to distant sites.

## Materials and methods

#### Generation of inducible cell lines

HEK293-AblPP, HEK293-Abl, and HEK293-AblKD cell lines were generated by using the T-Rex system (Invitrogen). In brief, the *AblPP*, *Abl*, or *AblKD* genes were cloned into the pcDNA5-FRT-TO plasmid; an HA tag was also attached at the C terminus of *AblPP*, *Abl*, or *AblKD* genes. pcDNA5-FRT-TO-AblPP, pcDNA5-FRT-TO-Abl, or pcDNA5-FRT-TO-AblKD, together with the Flp recombinase expression vector pOG44 were cotransfected into the HEK293-FRT cell line, which contains a single integrated FLP recombination target site and stably expresses the Tet repressor. The cells were then subjected to selection with 200 μg/ml of hygromycin and 15 μg/ml of blasticidin for 15 d. Single clones were then picked, amplified, and screened for induction efficiency.

#### Cell culture

The inducible cell lines were cultured in DME containing 10% FBS, 100 U penicillin/streptomycin, and 0.05% β-mercaptoethanol. Cells were routinely treated with 2 μg/ml Doxy at 37°C for protein induction. The PC3 cell line was cultured in RPMI 1640 media containing 10% FBS and 100 U penicillin/streptomycin.

#### Antibodies, chemicals, and plasmids

Mouse anti-Abl monoclonal antibody 8E9 was generated in our laboratory; mouse anti-HA was obtained from Covance; rabbit anti-ROCK1, rabbit anti-Rap1, and rabbit anti-C3G antibodies were purchased from Santa Cruz Biotechnology, Inc.; rabbit anti-Rho and rabbit anti–phospho-CrkII ($Tyr^{221}$) were obtained from Cell Signaling Technology; mouse anti–β1 integrin antibody P4C10 and the conformation-specific anti–β1 integrin antibody B44 were obtained from Millipore; mouse anti-CrkII was obtained from BD; mouse anti-actin, Doxy, hygromycin, blasticidin, and LPA were obtained from Sigma-Aldrich; imatinib was obtained from Novartis; and Fc-ephrin-A1 was obtained from R&D systems. The pcDNA3-Rap1V14 and pGEX-RalRBD plasmids were obtained from S. Shattil (University of California, San Diego [UCSD], La Jolla, CA); the pCDNA3-RhoV14 and -RhoN19 constructs were obtained from M. Ginsberg (UCSD); and the pcDNA3-CrkII221F construct was obtained from E. Pasquale (Burnham Institute for Medical Research, La Jolla, CA).

#### Immunoblotting and immunoprecipitation

Whole cell lysates were prepared in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 0.5 mM EDTA, 1 mM EGTA, and 1 mM DTT) plus protease inhibitor (from Sigma-Aldrich). Total protein was resolved by SDS-PAGE, transferred onto polyvinylidene fluoride membranes, blocked in 5% nonfat dry milk/TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20), and incubated with primary antibodies overnight at 4°C. Membranes were washed 3× for 10 min in TBST and incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. After three 10-min washings, membranes were incubated with ECL reagent or Femto Max Sensitivity Substrate (Thermo Fisher Scientific) and exposed to x-ray films.

Cells were lysed in NP-40 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.5 mM EDTA, 1 mM EGTA, and 1 mM DTT) plus protease inhibitor cocktail; 1 mg of whole cell lysates were used for immunoprecipitation. The immunoprecipitates were resolved by SDS-PAGE and target proteins were detected by immunoblotting.

### Cell adhesion assay

96-well plates were coated with fibronectin (Sigma-Aldrich) at concentrations from 0.1 to 20 μg/ml. Cells were brought into suspension by trypsinization, were seeded at $10^4$ cells per well, and were then allowed to adhere for 2 h at 37°C. Wells were then washed twice with serum-free DME/0.1% BSA, and adherent cells were fixed with 5% glutaraldehyde and then stained with crystal violet (0.1%). After extensive washing to remove the free dye, the cell-bound crystal violet was extracted with 0.5% Triton X-100, and absorbance was measured at 595 nm.

### Integrin activity assay

Integrin affinity was analyzed by flow cytometry. In brief, cells were stained with antibody B44, which recognizes an epitope present on active integrins, immediately upon harvest via a 15-min incubation at room temperature in PBS/3% BSA, followed by a 30-min incubation on ice. Cells were washed three times and stained with Alexa-conjugated goat anti–mouse antibody (Invitrogen) in PBS/BSA. Negative controls were stained with only a secondary antibody, whereas positive controls were labeled with monoclonal antibody P4C10, which binds to β1 integrins irrespective of activation state. The relative activation index was defined as $(F - F_0)/(F_{max} - F_0)$, where F is the mean fluorescent intensity (MFI) of B44 binding, $F_0$ is the MFI of negative control, and $F_{max}$ is the MFI of B44 binding in the presence of $Mn^{2+}$.

### Fluorescence microscopy and image acquisition

Cells were fixed with 4% paraformaldehyde/PBS for 15 min, permeabilized in 0.3% Triton X-100/PBS, and then stained with FITC-conjugated phalloidin (Invitrogen) to visualize actin. Stained cells were mounted in Prolong Gold antifade reagent with DAPI (Invitrogen). Images were taken at room temperature using a fluorescence microscope (Axioskop 2; Carl Zeiss, Inc.) equipped with a 40× (NA 0.75) Plan-Neofluar objective and a camera (Axiocam MRM; Carl Zeiss, Inc.). Brightness and contrast of the images were adjusted using Photoshop (Adobe).

### Cell detachment assay

HEK293-AblPP or HEK293-AblKD cells were cultured on poly-L-lysine–coated coverslips overnight, changed to serum-free media, and cultured for an additional 2 h. Cells were then treated with Doxy for 2 h to induce AblPP or AblKD protein. After that, cells were fixed with 4% paraformaldehyde for 15 min, washed with PBS, and observed under a phase-contrast microscope to count the rounding cells. At least 200 cells were counted under each condition.

### Pull-down assay of activated small GTPases

Cells were cultured overnight and treated with Doxy for 2 h to induce AblPP or AblKD expression. Cells were lysed and Rap1-GTP was pulled down using bacteria-purified GST-RalBD (RalGDS-binding domain; Franke et al., 1997). Rho-GTP was pulled down with bacteria-purified GST-Rho-BD (rhotekin Rho-binding domain; Hall and Nobes, 2000), and Rac-GTP was pulled down with GST-PBD as described previously (Jin and Wang, 2007). The pulled down proteins were subjected to immunoblotting with corresponding antibodies.

### siRNA and shRNA experiments

Sense and antisense RNA oligos corresponding to the target sequence for human *ROCK1* (GGUGAUUGGUAGAGGUGCA), and for *LacZ* (AACGTACGCGGAATACTTCGA) were synthesized and annealed by Applied Biosystems. The uniqueness of each individual target sequence was confirmed by a BLAST search of mouse genomic plus transcript database. Transfection of synthetic siRNA was performed using the Lipofectamine2000 reagent (Invitrogen) with standard procedures. 48 h after transfection, cells were collected and target protein levels were analyzed. Cotransfection of Cy3-labeled siRNA duplexes (Invitrogen) were performed to determine transfection efficiency when necessary.

We used MISSION TRC shRNA Target shRNA (Sigma-Aldrich) to knock down the endogenous Abl in PC3 cells. The functional sequence in the shRNA vector is CCGGGCTGAAATCCACCAAGCCTTTCTCGAGAAAGGCTTGGTGGATTTCAGCTTTTTG, which targets the human c-Abl gene sequence ($^{1462}$GCTGAAATCCACCAAGCCTTT$^{1482}$).

### Online supplemental material

Fig. S1 A shows the detection of CrkII–C3G complex before the induction of AblPP and the formation of an alternate CrkII–AblPP complex after the induction of AblPP in the HEK293-AblPP cells. Fig. S1 B shows that Rap1-GTP levels were not significantly altered during mouse embryo fibroblast spreading on fibronectin. The levels of Rap1-GTP were examined in embryo fibroblasts derived from the $Abl^{-/-}Arg^{-/-}$ mouse and those reconstituted for Abl expression using retroviral-mediated gene transfer. This result is consistent with previous findings that Abl does not affect the adhesion of mouse embryo fibroblasts to fibronectin. The online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801192/DC1.

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