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RIN1 Is an ABL Tyrosine Kinase Activator and a Regulator of Epithelial-Cell Adhesion and Migration

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Summary

Background: ABL tyrosine kinases control actin remodeling in development and in response to environmental stimuli. These changes affect cell adhesion, cell migration, and cell-cell contact. Little is known, however, about upstream mechanisms regulating ABL protein activation.
Results: We report that the RAS effector RIN1 is an activator of ABL tyrosine kinases. RIN1 expression in fibroblasts promotes the formation of membrane spikes; similar effects have been reported for ABL overexpression. RIN1 binds to the ABL SH3 and SH2 domains, and these interactions stimulate ABL2 catalytic activity. This leads to increased phosphorylation of CRK and CRKL, inhibiting these cytoskeletal regulators by promoting intramolecular over intermolecular associations. Activated RAS participates in a stable RAS-RIN1-ABL2 complex and stimulates the tyrosine kinase-activation function of RIN1. Deletion of the RAS binding domain (RBD) strongly stimulated the ABL2 activation function of RIN1, suggesting that RAS activation results from the relief of RIN1 autoinhibition. The ABL binding domain of RIN1 (RIN1-ABD) increased the activity of ABL2 immune complexes and purified RIN1-ABD-stimulated ABL2 kinase activity toward CRK. Mammary epithelial cells (MECs) from *Rin1*$^{-/-}$ mice showed accelerated cell adhesion and increased motility in comparison to wild-type cells. Knockdown of RIN1 in epithelial-cell lines blocked the induction of CRKL phosphorylation, confirming that RIN1 normally functions as an inhibitor of cell motility.
Conclusions: RIN1 is a directly binding ABL tyrosine kinase activator in cells as well as in a defined-component assay. In response to environmental changes, this novel signal pathway mediates actin remodeling associated with adhesion and migration of epithelial cells.

Introduction

The nonreceptor tyrosine kinases ABL1 (also known as c-Abl) and ABL2 (also known as Arg) are similar in structure and function [1]. Both proteins contain SH3, SH2, and tyrosine kinase (TK) domains; in each case, the domains exhibit >90% sequence identity. Both ABL proteins also have leukemogenic potential that is unleashed by chromosome translocations [2–4]. The resulting oncogenic fusion proteins always include the SH3, SH2, and TK domains.

ABL proteins regulate cytoskeleton remodeling (reviewed in [5, 6]) during adhesion, motility, and axon guidance. Consistent with this function, ABL proteins contain actin binding sites [7–9], localize to dynamic actin structures, and phosphorylate CRK and CRKL [10, 11], DOK1 [12], and other proteins controlling cytoskeleton dynamics [13]. Tyrosine phosphorylation of CRK/CRKL promotes intramolecular refolding that excludes interactions with CAS and blocks cell migration (reviewed in [14]).

Inactive ABL kinases are stabilized by intramolecular interactions including the following: (1) association between the SH3 domain and the SH2-TK linker region [15], (2) interactions of a short amino-terminal "cap" [16], and (3) contributions from an amino-terminal myristoyl group [17] and phospholipids [18]. ABL kinase activation is facilitated by autophosphorylation as well as by SRC-family kinase-mediated phosphorylation [19–21]. Intermolecular ABL binding partners that inhibit function include Abi1 [22], Abi2 [23], AAP1 [24], PAG [25], and RB1 [26]. Although overexpression of some genes can promote phosphorylation of ABL substrates in vivo [27, 28], there are no reports of direct ABL kinase activators.

RIN1 is a RAS-effector protein [29, 30] that binds to ABL1 [29] and BCR-ABL1 [31]. The RIN1-ABL1 interaction appears to involve the SH3 and SH2 domains of ABL1 [31, 32], but the mechanism of binding and the consequences of this association were previously not known. The expression of RIN1 protein is restricted [33] but includes some epithelial cells.

We report that RIN1 is a binding partner for ABL2 as well as for ABL1, that RIN1 phosphorylation by ABL is required for stable binding, that RIN1 functions to stimulate ABL2 activity in a RAS-responsive manner, and that the ABL binding domain of RIN1 can directly enhance ABL2-mediated phosphorylation of CRK. We also show that RIN1 promotes the cytoskeletal-remodeling properties of ABL proteins and serves in part to regulate epithelial-cell functions including adhesion and migration.

Results

RIN1 Functions through ABL Kinases to Induce Actin Remodeling

We introduced RIN1 into fibroblast cells, which normally have undetectable levels of this protein. When plated on fibronectin, RIN1-expressing cells showed a striking change in morphology (Figure 1A). We noted the appearance of microspike structures and a decrease in actin stress fibers, similar to changes seen after overexpression of ABL1 [12, 34]. RIN1-expressing cells also had a rounded-up morphology (Figure 1B; cell height: 8.3 ± 0.6 μm [vector], 14.8 ± 1.2 μm [RIN1]). Microspike formation was also observed when we expressed the ABL binding domain alone (RIN1-ABD, Fig-

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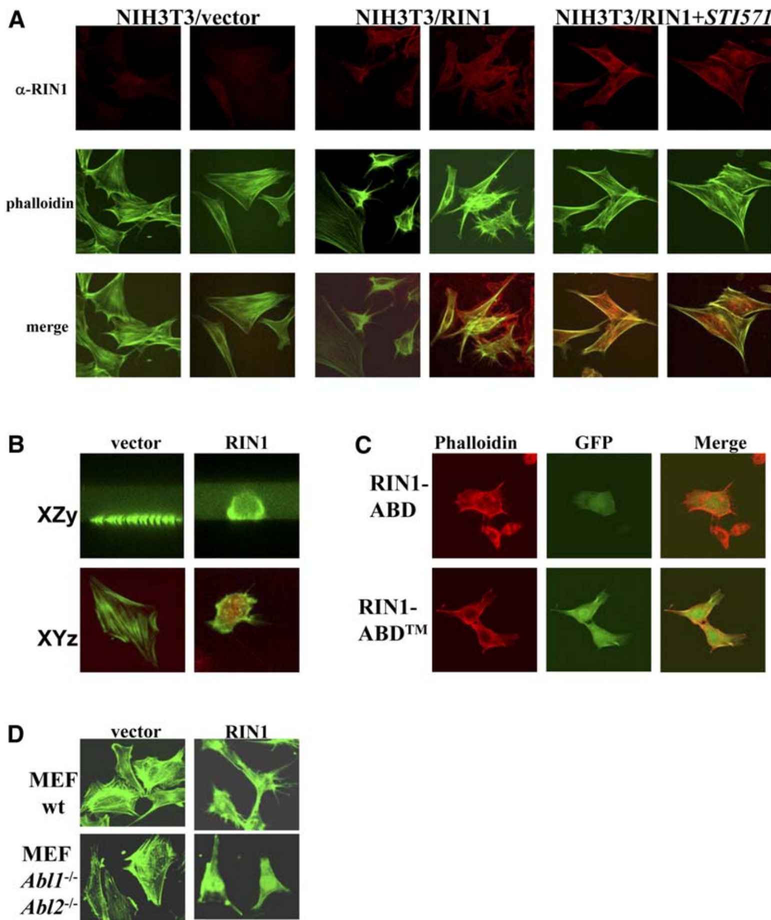

Figure 1. RIN1 Expression Promotes ABL-Dependent Actin Remodeling in Fibroblasts

(A) NIH3T3 cells, infected with a RIN1 or vector retrovirus and stained with phalloidin (green) and anti-RIN1 (red). RIN1 effects were blocked by STI571.

(B) At top, XZy confocal analysis, phalloidin (green) stain; at bottom, XYz confocal analysis and phalloidin (green) and anti-RIN1 (red) staining of the same cells as on top.

(C) NIH3T3 cells expressing the ABL binding domain alone (RIN1-ABD) or the triple Y/F mutation (RIN1-ABD^{TM}) from a green-fluorescent-protein (GFP) vector. The phalloidin stain was red in this experiment.

(D) MEFs, infected with a RIN1 or vector retrovirus and stained with phalloidin (green). RIN1 induced membrane spikes in wild-type but not in *Abl1*$^{-/-}$*Abl2*$^{-/-}$ MEFs.

ure 1C). These results are consistent with RIN1 activation of an ABL-mediated pathway. Treatment with STI571, a potent inhibitor of both ABL1 and ABL2, effectively blocked cell remodeling (Figure 1A) but had no effect on the morphology of NIH3T3 cells not expressing RIN1 (data not shown). To confirm that RIN1-induced remodeling was ABL dependent, we compared the effects of RIN1 expression in mouse embryo fibroblast (MEF) cells from wild-type and *Abl1*$^{-/-}$*Abl2*$^{-/-}$ animals [35]. The absence of ABL1 and ABL2 blocked the ability of RIN1 to induce cell shape changes (Figure 1D), although these cells remain responsive to ABL (Figure S1 in the Supplemental Data available with this article online).

RIN1 Is a Binding Partner of ABL Tyrosine Kinases

When ABL2 was immunoprecipitated from cell extracts, we observed copurification of RIN1 (Figure 2A, left), indicating a stable protein complex. This was confirmed by reciprocal analysis (RIN1 immunoprecipitation followed by ABL2 immunoblot; Figure 2A, right). We also detected an interaction between ectopically expressed RIN1 and ABL1 (Figure 2B, left) as well as between endogenous epithelial-cell RIN1 and ABL1 (Figure 2B, right). The RIN1-ABL1 coimmunoprecipitation appeared weaker than that of RIN1-ABL2, perhaps reflecting ABL1 distribution in both the cytoplasm and the nucleus.

Because RIN1 is phosphorylated by ABL1 in vitro [29] and by BCR-ABL1 in vivo [31], we tested whether RIN1 is subject to tyrosine phosphorylation by endogenous ABL proteins and whether this was required for RIN1-ABL binding. RIN1 ectopically expressed in wild-type, *Abl1*$^{-/-}$, or *Abl2*$^{-/-}$ MEF cells was tyrosine phosphorylated (Figure 3A), but RIN1 expressed in *Abl1*$^{-/-}$*Abl2*$^{-/-}$ cells had no detectable tyrosine phosphorylation. These findings suggested that RIN1 is phosphorylated by both ABL1 and ABL2. Because RIN1 tyrosine 36 conforms to both the general tyrosine-phosphorylation consensus (R/K-X_{2-3}-D/E-X_{2-3}-Y) and the ABL substrate motif (YxxP), we developed a RIN1pY^{36} phosphospecific antibody to monitor RIN1 phosphorylation by ABL1 and ABL2. Anti-RIN1pY^{36} was highly specific for tyrosine-phosphorylated RIN1 in cells coexpressing ABL2 (Figure 3B). In addition, the antibody recognized endogenous RIN1pY^{36} in KCL22 cells, a leukemia-derived cell line expressing BCR-ABL1 (Figure 3B), and in forebrain tissue, which has high levels of ABL2 and RIN1 [33, 35]. The identified RIN1pY^{36} band was absent from *Rin1*$^{-/-}$ tissue and showed reduced intensity in extracts from *Abl2*$^{-/-}$ forebrains (Figure 3C).

We next tested whether phosphorylation by ABL proteins is required for RIN1 binding. When cotransfected with RIN1, a catalytically inactive form of ABL2 (ABL2^{K319R}) gave only low levels of RIN1pY^{36} and weak

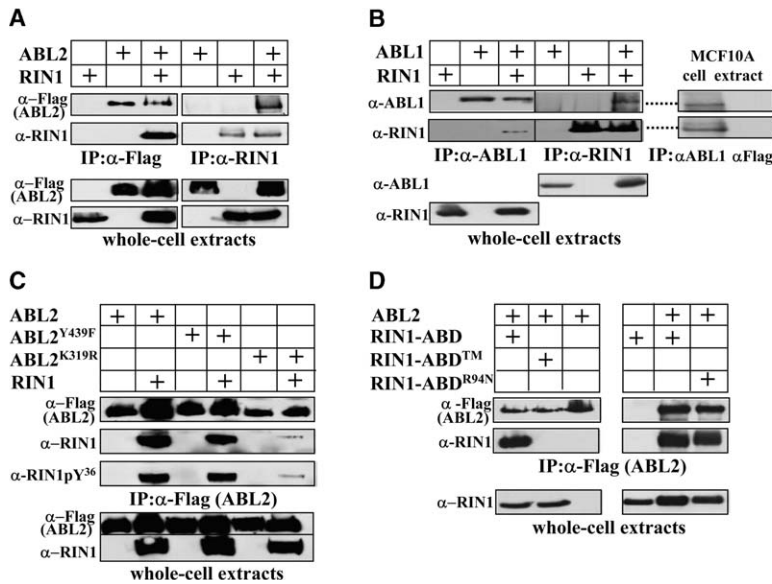

Figure 2. RIN1 Is Associated with ABL Proteins

(A) Anti-Flag (ABL2)-immunoprecipitated material was immunobloted with anti-RIN1 (left), and anti-RIN1-immunoprecipitated material was immunobloted with anti-Flag (right). These immunoblots used extracts from transfected 293T cells.

(B) RIN1 binding to ABL1 was assessed with anti-RIN1 or anti-ABL1 immunoprecipitation from extracts of transfected 293 cells. The right side shows immunoprecipitation of endogenous RIN1 with endogenous ABL1 from MCF10A cells when anti-ABL1 but not anti-Flag (control) was used.

(C) RIN1 tyrosine phosphorylation is required for ABL2 binding. RIN1 was efficiently coimmunoprecipitated with wild-type ABL2-Flag or a phosphorylation-site mutant ($ABL2^{Y439F}$) but not with a kinase inactive mutant ($ABL2^{K319R}$).

(D) RIN1 tyrosine phosphorylation is required for ABL2 binding. RIN1-ABD and an SH2 mutant ($RIN1\text{-}ABD^{R94N}$), but not a triple-tyrosine mutant ($RIN1\text{-}ABD^{TM}$), coimmunoprecipitated with ABL2-Flag. Whole-cell-extract immunoblots were used to confirm expression of transfected constructs.

ABL2-RIN1 binding (Figure 2C). The detectable $RIN1pY^{36}$ likely reflects the action of endogenous ABL1 and ABL2. Mutation of an activation-loop phosphorylation site (Y439) required for optimum kinase activity [21] had no effect on RIN1 phosphorylation or binding (Figure 2C).

We confirmed the requirement for RIN1 tyrosine phosphorylation in ABL2 binding through the use of a mutant form of the ABL binding domain (ABD). $RIN1\text{-}ABD^{TM}$ has three tyrosines mutated to phenylalanines (in the absence of Y^{36}, two other tyrosines within the ABD [Y^{121} and Y^{148}] are phosphorylated by BCR-ABL1 [31]). $RIN1\text{-}ABD^{TM}$ was impaired for binding to ABL2 (Figure 2D) and failed to induce membrane spikes in NIH3T3 cells (Figure 1C). A mutation (R94N) in the RIN1 SH2 domain, which mediates receptor tyrosine kinase interactions [36], had no effect on ABL binding.

These results are consistent with a protein-interaction model that is initiated by a weak association between the RIN1 proline-rich motif and the ABL SH3 domain and is followed by tyrosine phosphorylation of RIN1. Subsequent connection of this phosphotyrosine to the ABL SH2 domain produces a stable two-site association.

Stimulation of ABL2 Tyrosine Kinase Activity by RIN1

To determine whether RIN1 could influence the catalytic activity of ABL proteins, we examined cellular tyrosine-phosphorylation levels. Autophosphorylated ABL2 protein was easily detectable, and this was noticeably elevated when RIN1 was coexpressed in cells (Figure 4A). Phosphorylated RIN1 was also clearly evident (293T cells have extremely low levels of endogenous RIN1 [data not shown]). Several additional, unidentified bands also appeared in extracts coexpressing ABL2 and RIN1, indicating a broader induction of tyrosine phosphorylation. The new phosphotyrosine bands most likely reflect ABL2 stimulation because these bands were absent in extracts from cells transfected with $ABL2^{K319R}$, the kinase dead mutant (Figure 4A). We also found that RIN1 expression increased cellular tyrosine phosphorylation by $ABL2^{Y439F}$, a regulatory phosphorylation-site mutant of ABL2 (Figure 4A), providing evi-

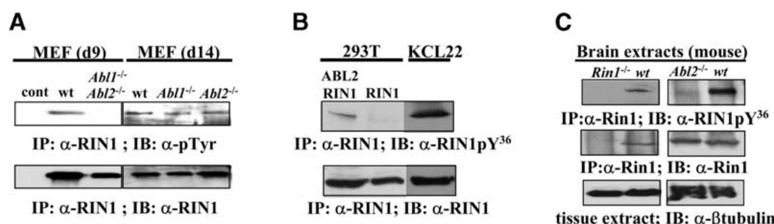

Figure 3. RIN1 Is Phosphorylated by ABL Kinases

(A) RIN1 was introduced into MEF cells and analyzed by anti-RIN1 immunoprecipitation followed by anti (α)-pTyr immunoblot (upper panel) or anti (α)-RIN1 immunoblot (lower panel). For $Abl1^{-/-}Abl2^{-/-}$ cells, matched day-9 MEFs were used because of early lethality [34]. Control cells were not infected with RIN1.

(B) Detection of ectopic phospho-RIN1 in transfected 293T cells (left) and endogenous phospho-RIN1 in BCR-ABL1-positive KCL22 cells (right) with $anti\text{-}RIN1pY^{36}$.

(C) Detection of endogenous $RIN1pY^{36}$ in forebrain tissue extracts prepared from adult mice of the indicated genotype. Rin1 was immunoprecipitated with anti-Rin1 and analyzed by immunoblot with $anti\text{-}RIN1pY^{36}$ or anti-Rin1. Brain extracts were normalized with an anti-β tubulin probe prior to immunoprecipitations.

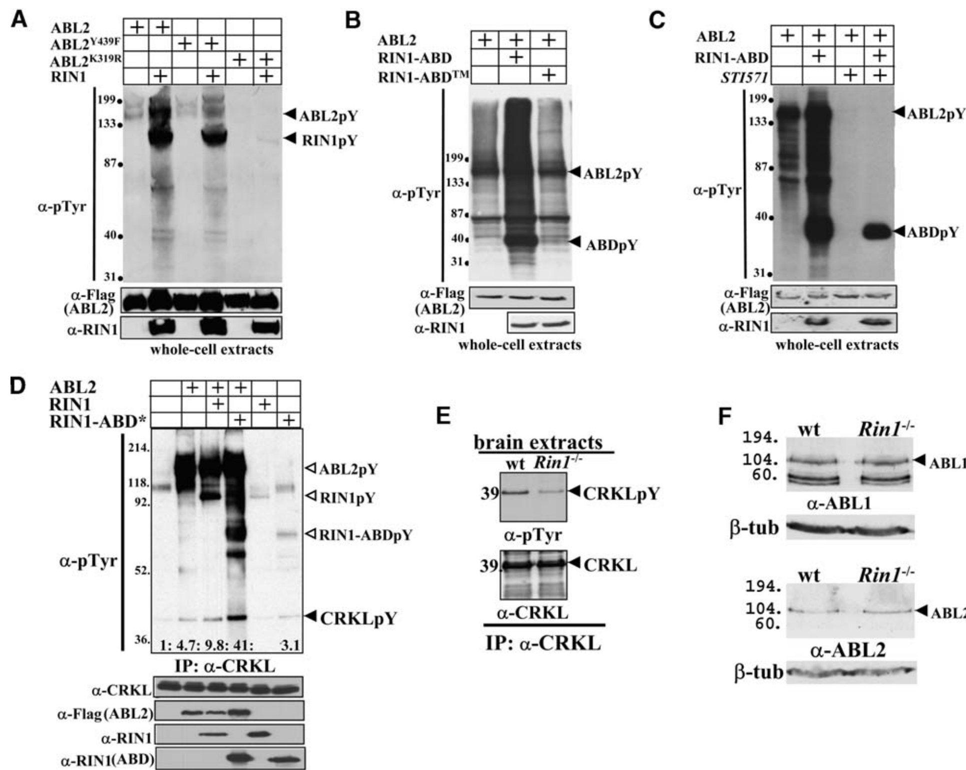

Figure 4. RIN1 Promotes ABL2 Kinase Activity

(A and B) Transfected 293T cell lysates were analyzed by immunoblot with anti-phosphotyrosine. ABL2 and RIN1 expression were confirmed by separate immunoblots (bottom panels). Note that in panel (B), gradient PAGE led to altered protein mobility.

(C) Analysis of cellular phosphotyrosine after treatment of cells with STI571 (10 μM).

(D) Endogenous CRKL was immunoprecipitated from lysates of cells transfected with the indicated constructs and was immunoblotted with anti-phosphotyrosine. * indicates an HTM tag, which reduces RIN-ABD migration in comparison to panels (B) and (C). Relative CRKL phosphorylation levels are indicated at the bottom of each lane. Immunoblots confirming expression and normalized CRKL levels are shown below.

(E) Endogenous CRKL was immunoprecipitated from forebrain extracts of wild-type (wt) and *Rin1*$^{-/-}$ mice and analyzed by anti-phosphotyrosine immunoblot. Below is a CRKL immunoblot for normalization.

(F) Analysis of ABL1 and ABL2 in wild-type and *Rin1*$^{-/-}$ brain extracts (several background bands are seen with this ABL1 antibody). Expression levels were normalized with a β-tubulin probe.

dence that RIN1 alters the catalytic properties of ABL2, at least in part, independently of this phosphoactivation.

Because all of the elements required for ABL binding reside in the ABD region of RIN1 (residues 1–295), we tested whether this domain alone could stimulate tyrosine kinase activity. Indeed, we detected strong stimulation of ABL2-mediated cellular phosphotyrosine by the ABD fragment of RIN1 (Figure 4B). However, RIN1-ABD^{TM}, which cannot bind to BCR-ABL1 [32] or ABL2 (this work), did not stimulate tyrosine kinase activity (Figure 4B). To confirm that immunoblot signals were due to ABL tyrosine kinase activity, the experiment was repeated with cells treated with STI571. Most of the antiphosphotyrosine signal was eliminated (Figure 4C), although, under these conditions, some phosphorylated RIN1-ABD was still detectable. Taken together, these results indicated that RIN1 promotes activation of ABL2 through a direct interaction.

We next examined the phosphorylation of CRKL, an ABL kinase substrate. Consistent with phosphotyrosine analyses of total cells, we observed a marked increase in tyrosine phosphorylation of endogenous CRKL when ABL2 was expressed with RIN1 or with RIN1-ABD (Figure 4D). An increase in tyrosine-phosphorylated CRKL was also noted in the sample that received only RIN1-ABD (compare lanes 1 and 6), reflecting stimulation of endogenous ABL proteins.

To determine whether RIN1 is a physiological regulator of CRKL phosphorylation, we analyzed mouse forebrain tissue, where RIN1 is expressed at highest levels [33]. We detected a band corresponding to tyrosine-phosphorylated CRKL, the intensity of which was markedly reduced in a *Rin1*$^{-/-}$ tissue sample (Figure 4E). The observed change was not due to reduced ABL1 or ABL2 expression (Figure 4F). These results further support a role for RIN1 in promoting the ABL kinase-mediated phosphorylation of CRKL.

Direct Activation of ABL Tyrosine Kinase Activity

We next performed in vitro kinase assays with a consensus ABL1/ABL2-substrate peptide [37] and ABL2 protein immunoprecipitated from cell lysates. Kinase activity was detected in material from ABL2 and ABL2^{Y439F} cell lysates, but ABL2^{K319R} yielded only background activity (Figure 5A). Coexpression of RIN1-

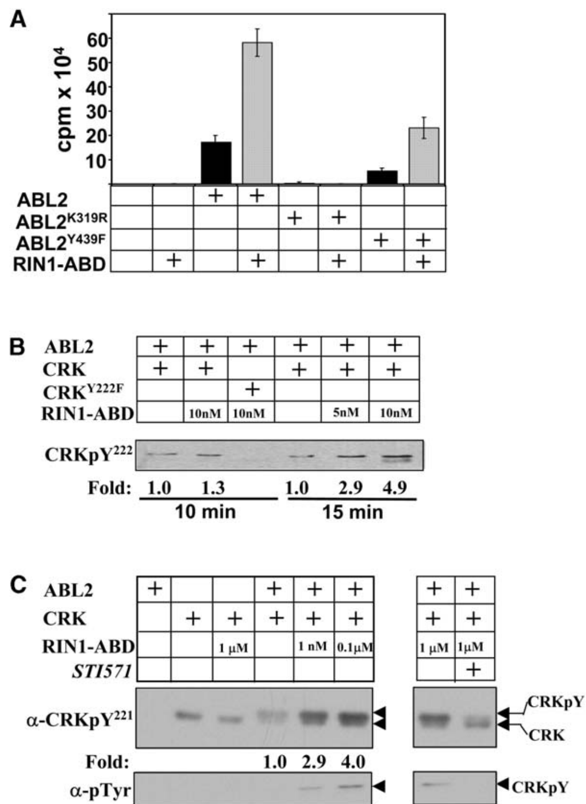

Figure 5. RIN1 Stimulates ABL2 In Vitro

(A) Samples immunoprecipitated with anti-Flag from cells transfected with ABL2-Flag (wt or mutant) and RIN1 constructs were used in kinase assays with ABL-substrate peptide. ABL2 levels in each assay were normalized by immunoblot. Error bars represent standard deviations calculated from results obtained with triplicate samples.

(B) Kinase assays with purified His_6-ABL2 (1.5 nM), GST-CRK or GST-CRK^{Y222F} (chicken) (17 nM), and RIN1-ABD-His_6 (5 nM or 10 nM). The RIN1-ABD effect is time dependent and concentration dependent.

(C) Kinase assays with purified His_6-ABL2 (1 nM), His_6-CRKII (human) (1 μM), and RIN1-ABD-His_6 (± STI571 [10 μM]). The normalized change in phospho-CRK is indicated below the immunoblot (Fold).

ABD enhanced the activity of ABL2 by 3.3-fold, suggesting that RIN1, which coimmunoprecipitates with ABL proteins, stimulates ABL2 activity. The $ABL2^{Y439F}$ mutant showed compromised basal activity but was stimulated (4.2-fold) by coexpression with RIN1-ABD (Figure 5A), consistent with the effects seen on total cellular tyrosine phosphorylation (Figure 4A) and indicating that RIN1-mediated stimulation is independent of $ABL2^{Y439}$ phosphorylation.

We next employed a purified-protein system to test whether RIN1 could *directly* stimulate ABL2. The kinase activity of ABL2 showed only a minor increase when RIN1-ABD was included in the peptide phosphorylation assay (data not shown). Using 10 nM of ABL2 and 10 μM of peptide, we observed only 1.4-fold (100 nM ABD) and 1.8-fold (1 μM ABD) increases in activity in comparison to assays with no ABD (n = 9 for each condition), suggesting that enhancement of ABL-substrate-peptide phosphorylation may require RIN1-induced changes in a cellular context.

To more closely approximate a natural ABL substrate, we used GST-CRK (chicken CRKII) (Figure 5B). Phosphorylation was specific for the ABL target site (Y^{222}, equivalent to Y^{221} of mammalian CRKII) because GST-CRK^{Y222F} was not phosphorylated under the same conditions. RIN1-ABD had a stimulatory effect on CRK phosphorylation (up to 4.9-fold), and this was both concentration- and time-dependent. Further assays were performed with human CRKII, with similar results (Figure 5C). The inhibitory effect of STI571 confirmed that the assay was detecting primarily ABL kinase activity (Figure 5C). These results suggest that ABL stimulation is an intrinsic property of RIN1, at least with regard to the CRK substrate. Cellular factors may participate in vivo to further modulate these effects.

RAS-RIN1-ABL2: A Signal-Transduction Pathway

We examined whether RIN1, a known RAS effector, can function as a physical conduit for signal transduction from RAS to ABL proteins. Expression of $HRAS^{G12V}$ (activated RAS), RIN1, and ABL2 resulted in the formation of a complex including all three proteins, as detected by immunoprecipitation of either ABL2 or HRAS (Figure 6A). In each case, the association of HRAS with ABL2 was dependent on the presence of RIN1. The formation of this stable complex is consistent with a pathway connecting RAS proteins with the cytoplasmic functions of ABL-family tyrosine kinases through RIN1.

The RIN1-ABD fragment appeared to be more potent than full-length RIN1 as a stimulator of ABL2 kinase activity (Figure 4D, Figure 6B), suggesting that the RAS binding domain of RIN1 might be a negative regulator of the ABL activation function. We therefore tested whether activated RAS could enhance the ABL2 stimulation capacity of RIN1. Indeed, the coordinated expression of RAS^{G12V} with RIN1 significantly enhanced the activation of ABL2 and cellular phosphotyrosine levels when compared with the results for RIN1 + ABL2 or RAS^{G12V} + ABL2 (Figure 6B). This observation is consistent with a signaling pathway from activated RAS through RIN1, leading to stimulation of ABL kinases. The specificity of enhanced phosphorylation was examined by direct evaluation of phospho-CRKL. The ABL2-mediated phosphorylation of endogenous CRKL was stimulated 7.5-fold by RIN1, and this was increased to 11-fold by coexpression of RAS^{G12V} (Figure 6C). The highest levels of CRKL phosphorylation (17-fold) were seen when the RIN1-ABD construct was used, consistent with a derepression role for RAS in the activation of RIN1 (Figure 6D).

RIN1 Regulates Adhesion and Migration

Cell adhesion and spreading require regulated activity of ABL tyrosine kinases [6, 12, 34]. To test whether RIN1 is involved in cell attachment, we examined the properties of mammary epithelial cells (MECs), which normally express RIN1. MECs from *Rin1*$^{-/-}$ mice showed higher levels of attachment to fibronectin than did MECs from wild-type mice after incubation for 15 min (5-fold) or 30 min (2-fold) (Figure 7A, left). Moreover, a striking morphology difference between *Rin1*$^{-/-}$ and wild-type

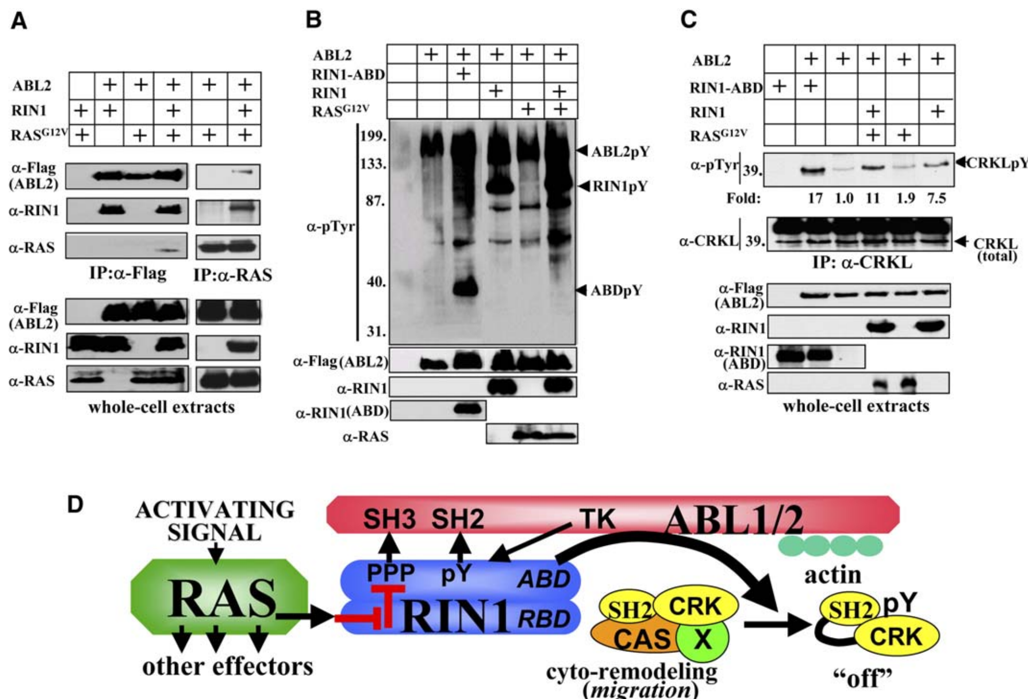

Figure 6. HRAS, RIN1, and ABL2 Form a Complex

(A) Transfected cell lysates were subjected to immunoprecipitation with anti-Flag (ABL2) or anti-RAS, and immunoblot for ABL2, RIN1, and RAS followed. Separate immunoblots (lower panels) confirmed expression.

(B) Lysates of transfected 293T cells were analyzed by immunoblot with anti-phosphotyrosine (upper panel) or antibodies to ectopically expressed proteins. Expected positions of phosphorylated ABL2-Flag, RIN1, and RIN1-ABD are indicated.

(C) Endogenous CRKL was immunoprecipitated and then immunoblotted with anti-phosphotyrosine to detect phospho-CRKL (the stimulation of CRKL phosphorylation, normalized to ABL2-only cells, is indicated below the immunoblot [Fold]). Anti-CRKL immunoblot was the immunoprecipitation control (intense band indicates position of IgG). Lower panels show immunoblots for ectopic proteins.

(D) Model for RIN1 activation of ABL proteins. RIN1 binds to ABL proteins through the following: (1) SH3 domain (ABL) binding to proline-rich domain (RIN1), (2) tyrosine phosphorylation of RIN1, and (3) SH2 domain (ABL) binding to pY (RIN1). RIN1 promotes tyrosine phosphorylation of ABL substrates. Phospho-CRK and -CRKL refold to accommodate an intramolecular SH2-pTyr pairing while disengaging from remodeling and promigration complexes that include CAS and other proteins (X). RAS appears to stimulate RIN1 through derepression. Some RIN1 effects may result from direct competition with other RAS effectors.

MECs was observed after attachment to fibronectin. *Rin1*$^{-/-}$ mutant cells showed extensive, striated, peripheral-actin networks (about 50% of total cell diameter), whereas wild-type cells showed much thinner cortical-actin staining (Figure 7B). Treatment of wild-type MECs with the ABL inhibitor STI571 enhanced attachment (Figure 7A, right) and resulted in peripheral-actin fiber network extensions (Figure 7B), consistent with a model in which ABL proteins normally inhibit or delay epithelial-cell attachment and spreading.

The extended peripheral-actin fiber networks seen in *Rin1*$^{-/-}$ cells may represent extensions used in cell migration. Migration is essential in development and wound healing but contributes to the malignancy of epithelial-cell-derived tumors. Indeed, we observed that *Rin1*$^{-/-}$ MECs migrated toward hepatocyte growth factor (HGF) at a higher rate than did wild-type MECs (Figure 7C). To confirm the role of RIN1 in regulating cell migration, we employed small interfering RNA (siRNA) to knock down RIN1 expression 10-fold in the human mammary epithelial-cell line MCF10A (Figure 7D). A negative-control siRNA at the same concentration had no effect (data not shown). RIN1 siRNA reduced CRKL phosphorylation levels (Figure 7D) and blocked CRKL phosphorylation induced by fibronectin (integrin activation) plus HGF (MET-receptor activation) (Figure 7E). Conversely, CRKL tyrosine phosphorylation in MCF10A cells was increased by expression of RIN1-ABD (2-fold, normalized) but not of RIN1-ABD^{TM} (Figure 7F). RIN1 knockdown also *enhanced* cell migration (Figure 7G), consistent with a positive role for an *unphosphorylated* CRKL complex in cell motility [38]. As expected, the ABL inhibitor STI571 reduced CRKL phosphorylation and promoted migration of MCF10A cells on fibronectin and HGF (Figure 7H), as it does in thyroid cells [39] and as the deletion of ABL genes does in fibroblasts [11].

Discussion

A Novel ABL Activation Pathway

RIN1 binding stimulates ABL2 activity, providing the only example of an ABL kinase activator that can function in a purified-protein system. RIN1 may activate ABL2 by inducing a conformational change that increases catalytic activity; binding to both the SH3 and SH2 domains of ABL would be predicted to relieve autoinhibitory aspects of the inactive ABL structure [6, 17]. This is consistent with the ability of RIN1 to rescue

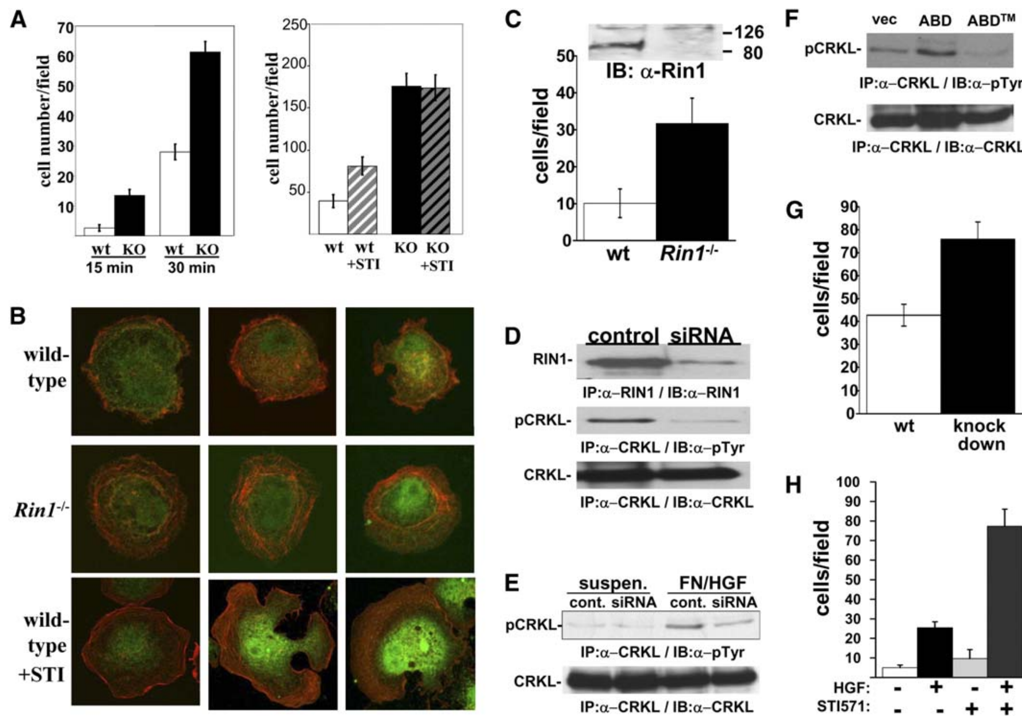

Figure 7. RIN1 Regulates ABL-Mediated Cytoskeletal Remodeling of Mammary Epithelial Cells

(A) Attachment of wild-type and *Rin1*$^{-/-}$ (KO) MECs to fibronectin-coated surfaces was measured at 15 min and 30 min after plating (left). In a separate experiment, a 30 min attachment was carried out in the presence or absence of STI571 (right).

(B) Wild-type and *Rin1*$^{-/-}$ MECs were attached to fibronectin-coated coverslips for 30 min, fixed and stained with anti-keritin (epithelial-cell marker, green) and phalloidin (red). Representative cells are presented.

(C) Migration of wild-type and *Rin1*$^{-/-}$ MECs through Boyden chambers toward HGF over 4 hr. Immunoblot (top) confirms Rin1 expression status.

(D) siRNA-mediated RIN1 knock down in MCF10A cells. The following are shown: at top, immunoblot of immunoprecipitated RIN1 from mock or RIN1-specific siRNA-treated cells (10-fold reduction); at middle, the same cells plated on fibronectin plus HGF for 30 min and an anti-pTyr immunoblot of immunoprecipitated CRKL (5.4-fold reduction of phospho-CRKL); at bottom, total CRKL levels (no change). Parallel treatment with control siRNA had no effect (data not shown).

(E) MCF10A cells were kept in suspension (lanes 1 and 2) or plated on fibronectin with HGF (FN/HGF, lanes 3 and 4). RIN1 knock down (lanes 2 and 4) blocked the induction of CRKL phosphorylation resulting from treatment with fibronectin and HGF.

(F) RIN1-ABD, RIN1-ABD^{TM}, or vector was expressed in MCF10A cells, and levels of phospho-CRKL were analyzed by immunoprecipitation and immunoblot.

(G) Migration of wild-type or RIN1 knockdown (siRNA) MCF10A cells through Boyden chamber over 16 hr.

(H) Migration of MCF10A cells, ± 10 μM STI571, through Boyden chamber over 4 hr.

the kinase activity of a regulatory phosphorylation-site mutant of ABL2 in vivo (this work) and the transforming activity of BCR-ABL1 with a corresponding mutation [31], and it suggests that RIN1 acts, at least in part, independently of SRC kinase-mediated ABL1^{Y412}/ABL2^{Y439} phosphorylation and activation [21].

In a purified-protein system, RIN1-ABD enhanced ABL2 kinase activity for CRK but not for a peptide substrate, suggesting that RIN1 may work in part by providing a surface that facilitates docking of ABL substrates such as CRK and CRKL. We observed the highest level of ABL kinase stimulation in vivo, where RIN1 might recruit binding partners or modifying enzymes that enhance ABL function. This is clearly not the sole mechanism for ABL activation, however, because *Rin1*$^{-/-}$ mice develop normally [33] whereas an *Abl1*$^{-/-}$*Abl2*$^{-/-}$ double mutation is lethal [35]. RIN1 signaling may instead be restricted to a limited number of cell types and a select set of cell stimulations.

Deletion of the RAS binding domain (RBD) of RIN1 resulted in a heightened ability to stimulate ABL2. This demonstrates that the structural determinants for ABL activation are within the ABD region (amino acids 1–295) and suggests that the RBD behaves as a negative regulator that is derepressed by RAS binding (Figure 6D). An analogous deletion of the RBD of RAF1 results in constitutively activated RAF (reviewed in [40]).

Regulation of Cytoskeletal Remodeling and Epithelial-Cell Function by RIN1

ABL proteins have a particularly important role in epithelial biology. Disruption of both mouse *Abl* genes causes severely malformed neuroepithelial structures with abnormal actin networks [35]. Mutations in fly *Abl* lead to excess cortical actin and defects in epithelial architecture [41, 42]. In mammalian epithelial cells, the ABL substrate CRK regulates adherens junctions and epithelial-mesenchymal transitions [43]. *Rin1*$^{-/-}$ MECs showed rapid cell-substratum adhesion, enhanced peripheral-actin fiber networks, and increased cell motil-

ity. These results suggest that, as a positive regulator of ABL-mediated cell remodeling, RIN1 normally favors cell-cell interactions and may function to inhibit epithelial-to-mesenchymal transitions such as those associated with tumor progression and metastasis. Indeed, RIN1 appears to be downregulated in human breast tumor cells [44].

The remodeling that accompanies epithelial-cell adhesion and motility likely involves the adaptor proteins CRK and CRKL, known ABL substrates [11] that coordinate the functions of CAS and other proteins. Tyrosine phosphorylation of CRK/CRKL leads to an *intra*molecular SH2-pY interaction that disrupts *inter*molecular interactions with CAS and inhibits cell motility [45] (Figure 6D). Conversely, CRK^{Y221F} increases both CAS binding and cell motility [38]. We observed reductions in CRKL phosphorylation in $Rin1^{-/-}$ tissues and cells and in siRNA-treated cells, both of which correlated with increased cell motility. We also found a stimulation of endogenous CRKL phosphorylation by RIN1 in cells expressing ABL2 and enhanced CRK phosphorylation by RIN1-stimulated ABL2 in vitro. These data suggest that RIN1 functions, at least in part, through regulation of ABL-mediated CRK/CRKL phosphorylation. Attenuation mechanisms for RAS-RIN1-ABL signaling may include dephosphorylation of RIN1 or ABL [46], 14-3-3-mediated redistribution of RIN1 [30], or ubiquitination-mediated protein degradation of ABL [47].

RIN1 expression is highest in mature forebrain neurons, where ABL2 is also highly expressed. $Rin1^{-/-}$ mice display a striking *enhancement* of long-term potentiation (LTP) and learning [33], functions that are in part dependent on actin remodeling [48], suggesting that RIN1 negatively regulates ABL-mediated neuronal plasticity. In hematopoietic cells, BCR-ABL1 is a potent oncoprotein that works in part through cytoskeletal remodeling [2, 7, 49] correlated with CRKL phosphorylation. The ability of RIN1 to promote leukemogenesis by BCR-ABL1 [31] and the correlation of RIN1 expression with leukemogenesis [50] are consistent with an ABL activation function. It should be noted that RIN1 also encodes a RAB5-exchange-factor domain that activates RAB-mediated endocytosis [36, 51], another function that requires cytoskeletal remodeling. The RAB5-GEF domain of RIN1 is not present, however, in the RIN1-ABD fragment that can activate ABL.

The properties of RIN1 establish it as a novel RAS effector regulating ABL tyrosine kinase activity and cytoskeletal remodeling. These findings should provide a greater understanding of stimulus-induced actin reorganization during epithelial-cell transitions, as well as insights into the tumorigenic and neurobiological properties of RAS and ABL proteins.

Supplemental Data

Supplemental Data including Supplemental Experimental Procedures and one figure can be found online at: http://www.current-biology.com/cgi/content/full/15/9/815/DC1/.

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References

1. Pendergast, A.M. (2002). The Abl family kinases: Mechanisms of regulation and signaling. Adv. Cancer Res. *85*, 51–100.
2. Advani, A.S., and Pendergast, A.M. (2002). Bcr-Abl variants: Biological and clinical aspects. Leuk. Res. *26*, 713–720.
3. Cazzaniga, G., Tosi, S., Aloisi, A., Giudici, G., Daniotti, M., Pioltelli, P., Kearney, L., and Biondi, A. (1999). The tyrosine kinase abl-related gene ARG is fused to ETV6 in an AML-M4Eo patient with a t(1;12)(q25;p13): Molecular cloning of both reciprocal transcripts. Blood *94*, 4370–4373.
4. Iijima, Y., Ito, T., Oikawa, T., Eguchi, M., Eguchi-Ishimae, M., Kamada, N., Kishi, K., Asano, S., Sakaki, Y., and Sato, Y. (2000). A new ETV6/TEL partner gene, ARG (ABL-related gene or ABL2), identified in an AML-M3 cell line with a t(1;12)(q25;p13) translocation. Blood *95*, 2126–2131.
5. Hernandez, S.E., Krishnaswami, M., Miller, A.L., and Koleske, A.J. (2004). How do Abl family kinases regulate cell shape and movement? Trends Cell Biol. *14*, 36–44.
6. Woodring, P.J., Hunter, T., and Wang, J.Y. (2003). Regulation of F-actin-dependent processes by the Abl family of tyrosine kinases. J. Cell Sci. *116*, 2613–2626.
7. McWhirter, J.R., and Wang, J.Y. (1993). An actin-binding function contributes to transformation by the Bcr-Abl oncoprotein of Philadelphia chromosome-positive human leukemias. EMBO J. *12*, 1533–1546.
8. Van Etten, R.A., Jackson, P.K., Baltimore, D., Sanders, M.C., Matsudaira, P.T., and Janmey, P.A. (1994). The COOH terminus of the c-Abl tyrosine kinase contains distinct F- and G-actin binding domains with bundling activity. J. Cell Biol. *124*, 325–340.
9. Wang, Y., Miller, A.L., Mooseker, M.S., and Koleske, A.J. (2001). The Abl-related gene (Arg) nonreceptor tyrosine kinase uses two F-actin- binding domains to bundle F-actin. Proc. Natl. Acad. Sci. USA *98*, 14865–14870.
10. Escalante, M., Courtney, J., Chin, W.G., Teng, K.K., Kim, J.I., Fajardo, J.E., Mayer, B.J., Hempstead, B.L., and Birge, R.B. (2000). Phosphorylation of c-Crk II on the negative regulatory Tyr222 mediates nerve growth factor-induced cell spreading and morphogenesis. J. Biol. Chem. *275*, 24787–24797.
11. Kain, K.H., and Klemke, R.L. (2001). Inhibition of cell migration by Abl family tyrosine kinases through uncoupling of Crk-CAS complexes. J. Biol. Chem. *276*, 16185–16192.
12. Woodring, P.J., Meisenhelder, J., Johnson, S.A., Zhou, G.L., Field, J., Shah, K., Bladt, F., Pawson, T., Niki, M., Pandolfi, P.P., et al. (2004). c-Abl phosphorylates Dok1 to promote filopodia during cell spreading. J. Cell Biol. *165*, 493–503.
13. Van Etten, R.A. (1999). Cycling, stressed-out and nervous: Cellular functions of c-Abl. Trends Cell Biol. *9*, 179–186.
14. Chodniewicz, D., and Klemke, R.L. (2004). Regulation of integrin-mediated cellular responses through assembly of a CAS/Crk scaffold. Biochim. Biophys. Acta *1692*, 63–76.
15. Barila, D., and Superti-Furga, G. (1998). An intramolecular SH3-domain interaction regulates c-Abl activity. Nat. Genet. *18*, 280–282.
16. Pluk, H., Dorey, K., and Superti-Furga, G. (2002). Autoinhibition of c-Abl. Cell *108*, 247–259.
17. Nagar, B., Hantschel, O., Young, M.A., Scheffzek, K., Veach, D., Bornmann, W., Clarkson, B., Superti-Furga, G., and Kuriyan, J. (2003). Structural Basis for the Autoinhibition of c-Abl Tyrosine Kinase. Cell *112*, 859–871.

18. Plattner, R., Irvin, B.J., Guo, S., Blackburn, K., Kazlauskas, A., Abraham, R.T., York, J.D., and Pendergast, A.M. (2003). A new link between the c-Abl tyrosine kinase and phosphoinositide signalling through PLC-gamma1. Nat. Cell Biol. *5*, 309–319.
19. Plattner, R., Kadlec, L., DeMali, K.A., Kazlauskas, A., and Pendergast, A.M. (1999). c-Abl is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF. Genes Dev. *13*, 2400–2411.
20. Brasher, B.B., and Van Etten, R.A. (2000). c-Abl has high intrinsic tyrosine kinase activity that is stimulated by mutation of the Src homology 3 domain and by autophosphorylation at two distinct regulatory tyrosines. J. Biol. Chem. *275*, 35631–35637.
21. Tanis, K.Q., Veach, D., Duewel, H.S., Bornmann, W.G., and Koleske, A.J. (2003). Two distinct phosphorylation pathways have additive effects on abl family kinase activation. Mol. Cell. Biol. *23*, 3884–3896.
22. Shi, Y., Alin, K., and Goff, S.P. (1995). Abl-interactor-1, a novel SH3 protein binding to the carboxy-terminal portion of the Abl protein, suppresses v-abl transforming activity. Genes Dev. *9*, 2583–2597.
23. Dai, Z., and Pendergast, A.M. (1995). Abi-2, a novel SH3-containing protein interacts with the c-Abl tyrosine kinase and modulates c-Abl transforming activity. Genes Dev. *9*, 2569–2582.
24. Zhu, J., and Shore, S.K. (1996). c-ABL tyrosine kinase activity is regulated by association with a novel SH3-domain-binding protein. Mol. Cell. Biol. *16*, 7054–7062.
25. Wen, S.T., and Van Etten, R.A. (1997). The PAG gene product, a stress-induced protein with antioxidant properties, is an Abl SH3-binding protein and a physiological inhibitor of c-Abl tyrosine kinase activity. Genes Dev. *11*, 2456–2467.
26. Welch, P.J., and Wang, J.Y. (1993). A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle. Cell *75*, 779–790.
27. Barila, D., Mangano, R., Gonfloni, S., Kretzschmar, J., Moro, M., Bohmann, D., and Superti-Furga, G. (2000). A nuclear tyrosine phosphorylation circuit: c-Jun as an activator and substrate of c-Abl and JNK. EMBO J. *19*, 273–281.
28. Miyoshi-Akiyama, T., Aleman, L.M., Smith, J.M., Adler, C.E., and Mayer, B.J. (2001). Regulation of Cbl phosphorylation by the Abl tyrosine kinase and the Nck SH2/SH3 adaptor. Oncogene *20*, 4058–4069.
29. Han, L., Wong, D., Dhaka, A., Afar, D., White, M., Xie, W., Herschman, H., Witte, O., and Colicelli, J. (1997). Protein binding and signaling properties of RIN1 suggest a unique effector function. Proc. Natl. Acad. Sci. USA *94*, 4954–4959.
30. Wang, Y., Waldron, R.T., Dhaka, A., Patel, A., Riley, M.M., Rozengurt, E., and Colicelli, J. (2002). The RAS effector RIN1 directly competes with RAF and is regulated by 14-3-3 proteins. Mol. Cell. Biol. *22*, 916–926.
31. Afar, D.E., Han, L., McLaughlin, J., Wong, S., Dhaka, A., Parmar, K., Rosenberg, N., Witte, O.N., and Colicelli, J. (1997). Regulation of the oncogenic activity of BCR-ABL by a tightly bound substrate protein RIN1. Immunity *6*, 773–782.
32. Lim, Y.M., Wong, S., Lau, G., Witte, O., and Colicelli, J. (2000). BCR/ABL inhibition by an escort/phosphatase fusion protein. Proc. Natl. Acad. Sci. USA *97*, 12233–12238.
33. Dhaka, A., Costa, R.M., Hu, H., Irvin, D.K., Patel, A., Kornblum, H.I., Silva, A.J., O'Dell, T.J., and Colicelli, J. (2003). The Ras effector Rin1 modulates the formation of aversive memories. J. Neurosci. *23*, 748–757.
34. Woodring, P.J., Litwack, E.D., O'Leary, D.D., Lucero, G.R., Wang, J.Y., and Hunter, T. (2002). Modulation of the F-actin cytoskeleton by c-Abl tyrosine kinase in cell spreading and neurite extension. J. Cell Biol. *156*, 879–892.
35. Koleske, A.J., Gifford, A.M., Scott, M.L., Nee, M., Bronson, R.T., Miczek, K.A., and Baltimore, D. (1998). Essential roles for the Abl and Arg tyrosine kinases in neurulation. Neuron *21*, 1259–1272.
36. Barbieri, M.A., Kong, C., Chen, P.I., Horazdovsky, B.F., and Stahl, P.D. (2003). The SRC homology 2 domain of Rin1 mediates its binding to the epidermal growth factor receptor and regulates receptor endocytosis. J. Biol. Chem. *278*, 32027–32036.
37. Voss, J., Posern, G., Hannemann, J.R., Wiedemann, L.M., Turhan, A.G., Poirel, H., Bernard, O.A., Adermann, K., Kardinal, C., and Feller, S.M. (2000). The leukaemic oncoproteins Bcr-Abl and Tel-Abl (ETV6/Abl) have altered substrate preferences and activate similar intracellular signalling pathways. Oncogene *19*, 1684–1690.
38. Takino, T., Tamura, M., Miyamori, H., Araki, M., Matsumoto, K., Sato, H., and Yamada, K.M. (2003). Tyrosine phosphorylation of the CrkII adaptor protein modulates cell migration. J. Cell Sci. *116*, 3145–3155.
39. Frasca, F., Vigneri, P., Vella, V., Vigneri, R., and Wang, J.Y. (2001). Tyrosine kinase inhibitor STI571 enhances thyroid cancer cell motile response to Hepatocyte Growth Factor. Oncogene *20*, 3845–3856.
40. Morrison, D.K., and Cutler, R.E. (1997). The complexity of Raf-1 regulation. Curr. Opin. Cell Biol. *9*, 174–179.
41. Grevengoed, E.E., Fox, D.T., Gates, J., and Peifer, M. (2003). Balancing different types of actin polymerization at distinct sites: Roles for Abelson kinase and Enabled. J. Cell Biol. *163*, 1267–1279.
42. Baum, B., and Perrimon, N. (2001). Spatial control of the actin cytoskeleton in Drosophila epithelial cells. Nat. Cell Biol. *3*, 883–890.
43. Lamorte, L., Royal, I., Naujokas, M., and Park, M. (2002). Crk adapter proteins promote an epithelial-mesenchymal-like transition and are required for HGF-mediated cell spreading and breakdown of epithelial adherens junctions. Mol. Biol. Cell *13*, 1449–1461.
44. Chen, C.R., Kang, Y., and Massague, J. (2001). Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program. Proc. Natl. Acad. Sci. USA *98*, 992–999.
45. Feller, S.M. (2001). Crk family adaptors-signalling complex formation and biological roles. Oncogene *20*, 6348–6371.
46. Cong, F., Spencer, S., Cote, J.F., Wu, Y., Tremblay, M.L., Lasky, L.A., and Goff, S.P. (2000). Cytoskeletal protein PSTPIP1 directs the PEST-type protein tyrosine phosphatase to the c-Abl kinase to mediate Abl dephosphorylation. Mol. Cell *6*, 1413–1423.
47. Echarri, A., and Pendergast, A.M. (2001). Activated c-Abl is degraded by the ubiquitin-dependent proteasome pathway. Curr. Biol. *11*, 1759–1765.
48. Sheng, M., and Kim, M.J. (2002). Postsynaptic signaling and plasticity mechanisms. Science *298*, 776–780.
49. Salgia, R., Li, J.L., Ewaniuk, D.S., Pear, W., Pisick, E., Burky, S.A., Ernst, T., Sattler, M., Chen, L.B., and Griffin, J.D. (1997). BCR/ABL induces multiple abnormalities of cytoskeletal function. J. Clin. Invest. *100*, 46–57.
50. Morikawa, J., Li, H., Kim, S., Nishi, K., Ueno, S., Suh, E., Dougherty, E., Shmulevich, I., Shiku, H., Zhang, W., et al. (2003). Identification of signature genes by microarray for acute myeloid leukemia without maturation and acute promyelocytic leukemia with t(15;17)(q22;q12)(PML/RARalpha). Int. J. Oncol. *23*, 617–625.
51. Tall, G.G., Barbieri, M.A., Stahl, P.D., and Horazdovsky, B.F. (2001). Ras-activated endocytosis is mediated by the Rab5 guanine nucleotide exchange activity of RIN1. Dev. Cell *1*, 73–82.