Abstract

Platelet-derived Growth Factor Receptor Beta Activates Abl2 through Direct Binding and Phosphorylation

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The catalytic activity of Abl family kinases is tightly regulated in cells by a complex set of intra- and intermolecular interactions and post-translational modifications. Abl family kinases are activated by diverse cellular stimuli, including receptor tyrosine kinase signaling. For example, the platelet-derived growth factor receptor beta (PDGFRβ) is a potent activator of Abl family kinases. However, the molecular mechanism by which PDGFRβ engages and activates Abl family kinases is not known. We find that the Abl2 Src Homology 2 (SH2) domain directly binds to phospho–tyrosine Y771 in the PDGFRβ cytoplasmic domain. PDGFRβ directly phosphorylates Abl2 N-terminal half on multiple novel sites including Y116, Y139 and Y161 within the SH3 domain, and Y299, Y303 and Y310 on the kinase domain. Y116, Y161, Y272 and Y310 are all located at or near the SH3/SH2-kinase linker interface, which helps maintain Abl family kinases in an auto-inhibited conformation. We found that PDGFRβ-mediated phosphorylation of Abl2 *in vitro* activates Abl2 kinase activity, but mutation of these four tyrosine (Y116, Y161, Y272 and Y310) to phenylalanine abrogated PDGFRβ-mediated activation of Abl2. These findings reveal how the PDGFRβ engages and phosphorylates Abl2 and how this leads to activation of the kinase, providing a framework to understand how growth factor receptors engage and activate Abl family kinases. Finally, the biological importance of PDGFR-

mediated activation on Abl2 is unclear. Our lab recently showed that Abl2 directly binds and phosphorylates the integrin β1 cytoplasmic tail. Here, I provide initial findings suggesting that PDGFR-mediated activation on Abl2 promotes Abl2-integrin β1 interaction and β1 phosphorylation by Abl2. This process also inhibits talin-integrin interaction. Since talin is the most important integrin activator, Abl2 may regulate talin-integrin interaction, and potentially acts as an integrin inactivator. Abl2 acting downstream of PDGFR to regulate integrin activation may provide a mechanism to allow PDGFR to crosstalk with integrin to regulate many processes including cancer metastasis, angiogenesis and embryonic development.

Platelet-derived Growth Factor Receptor Beta Activates Abl2 through Direct Binding and Phosphorylation

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For my family

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Table of Content

CHAPTER 1- A MECHANISTIC OVERVIEW OF ABL FAMILY NON-RECEPTOR TYROSINE KINASE.

Introduction

The Abelson (ABL) family of nonreceptor tyrosine kinases, comprised of Abl1 and Abl2 in vertebrates, translate diverse extracellular signals to regulate cell proliferation, survival, migration and invasion. Abl1 was first identified as an oncogene for the development of leukemia induced by Abelson murine leukemia virus (A-MuLV) or chromosome translocation that results in expression of the Bcr-Abl1 fusion protein (1-3). In addition to leukemia, recent studies have demonstrated Abl family kinase's role in solid tumor development (4-7), neurodegenerative disease (8,9) and microbial pathogenesis (10-12). Understanding how Abl kinases are regulated and what signaling pathways they modulate is crucial in developing therapies for these diseases.

The catalytic activity of Abl family kinases is important in promoting actin-based cell edge protrusion, facilitating endocytosis and phagocytosis, mediating DNA damage responses, and regulating cell survival and proliferation in a variety of cell contexts. These processes play essential roles in the development and function of the cardiovascular, brain, and immune systems, among others (12-23). This chapter will focus on the molecular mechanism of Abl family kinase activation and regulation.

Conserved structural organization and cellular distribution of Abl family kinases

Abl kinases have been identified in all metazoans. Vertebrates express two closely related paralogs, Abl1 and Abl2, with highly conserved SH3-SH2-Kinase cassette domain structure (Fig 1.1) (24,25). There are two major isoforms of both Abl1 and Abl2 including the non myristoylated isoform 1a, and the myristoylated isoform 1b. The N-terminal SH3-SH2-kinase domains of Ab1 and Abl2 share over 90% sequence identity (Fig1.1) (26). Although Src kinase has a domain organization similar to the N terminal half of Abl family kinases, they only share 52% identity in their kinase domains, and 37% in their SH3 and SH2 domains (Fig1.1) (27). Abl1 and Abl2 have overlapping function in particular cellular contexts, but possess unique roles due to their distinct C-terminal half domain structure. For example, Abl1 tends to localize to the nucleus as it has a DNA-binding domain, three nuclear localization signals and a nuclear export signal in its C-terminal half (24,28). On the other hand, Abl2 tends to localize at the cell periphery, which is enriched with cell cytoskeleton, due to its microtubule and actin binding domain in its C-terminus (Fig1.1) (29-31). Recent studies in our lab show Abl2 not only binds growing microtubules and promotes microtubule polymerization and stability, but also recruited to actin waves through cytoskeletal interactions (30,31).

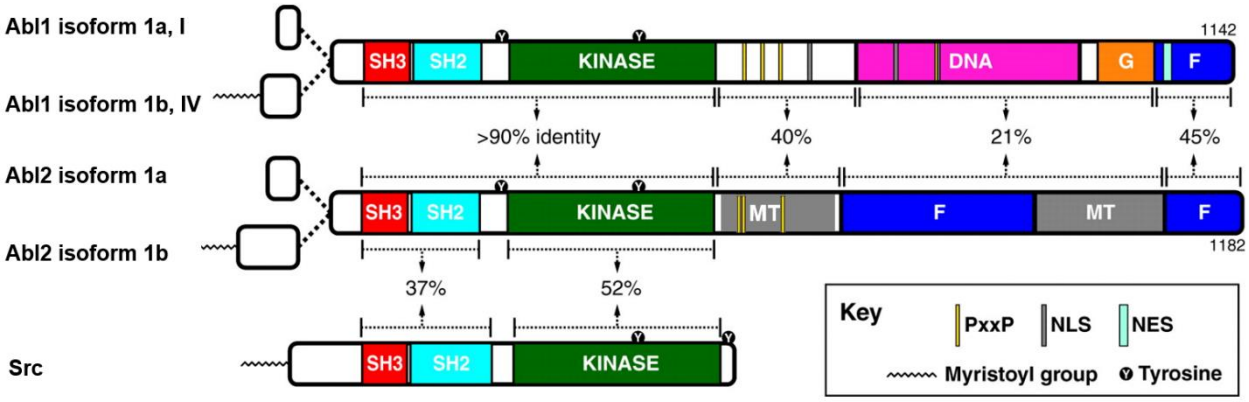


Figure 1.1 Domain Organization of Abl family kinases

Domain organization and sequence comparison of Abl1, Abl2 and Src. Abl1, Abl2 and Src share high sequence similarity in the N-terminal half SH3-SH2-SH1(Tyrosine Kinase) cassette. Abl1 and Abl2 each possess a unique C-terminal domain composition, resulting in different cellular localization and function. Abl1 and Abl2 both have PxxP motifs (yellow strips). Abl1 has nuclear localization sequences (NLS, gray stripes), a DNA-binding region (DNA, magenta) and a nuclear export sequence (NES, light-blue stripe). Additionally, Abl1 has G-actin (G, orange) and F-actin (F, purple)-binding domains. Abl2 has two F-actin-binding domains and two microtubule binding domains (MT, gray). Figure adapted from (25).

The SH3 domain

SH3 domain binds to proline-rich peptide with a P-X-X-P consensus motif (P is proline and X is any amino acid), which adopts polyproline type II helical

conformation to mediate both intermolecular and intramolecular interactions (32-36). Interestingly, the crystal structure of Abl1 SH3 domain with bound ligand was the first SH3 domain structure revealing how SH3 domain mediate protein-protein interaction (36). It has since been reported that many PXXP containing proteins are able to bind and regulate Abl family kinases (37).

The SH2 domain

SH2 domain bind to phosphotyrosine-containing peptides with sequence specificity. Different SH2 domains have different preferences regarding the three to six amino acid residues downstream from the phosphorylated tyrosine (38,39). The Abl family kinase SH2 domain binds preferentially to the consensus sequence of pY-X-X-P/L (pY is phosphotyrosine) (40,41). Our lab had shown that Ab1 and Abl2 SH2 domains have significantly different (over 10-fold) binding affinities for cortactin, mediated through a two-residue difference in their SH2 domain sequence (42). Abl family kinases SH2 domain had been reported to bind to many different proteins These interactions play important role in modulating Abl kinase activity (37,43,44).

The kinase domain

Abl family kinases are tyrosine kinase that specifically catalyze the transfer of phosphate group from ATP on to the side chain hydroxyl of tyrosine residue. Protein kinases play important role in signal transduction because phosphorylation of target proteins can modulate their function in many different ways. For example, phosphorated tyrosine residues in different receptors serve

as docking sites to recruit multiple SH2 domain-containing signaling proteins (as discussed above for SH2 domain interaction). Abl family kinase activity is also regulated by different phosphorylation events (details of kinase regulation will be discussed in next section) (45-47). Besides autophosphorylation, Abl family kinases have been reported to phosphorylated many different substrates including cortactin, Crk, integrin and p190RhoGAp (44,48-50).

Mechanistic overview of the structure and dynamic regulation of Abl family kinases

Leukemia and tyrosine kinase inhibitors

The catalytic activity of Abl family kinases is tightly regulated through a complex set of intermolecular and intramolecular interaction and post-translational modifications (25-29). Inappropriate kinase regulation drive leukemia development and promote solid tumor progression (4-6,23,51,52). One of the best examples is the discovery of breakpoint cluster region (Bcr)-Abl1 fusion protein, which is considered as the hallmark oncogenic protein that drive over 90% of Chronic Myelogenous Leukemia (CML) and a smaller subset of Acute Lymphocytic Leukemia (ALL) (3,53). Bcr-Abl1 is a fusion protein resulting from chromosomal translocation between chromosome 9 and 22 known as Philadelphia chromosome (3). Several additional Abl1 and Abl2 fusion oncoproteins since been discovered including Etv6-Abl1 and Etv6-Abl2 (54). One common feature of these fusion Abl family kinases is its deregulated, constitutively-active tyrosine kinase activity that drive multiple signaling pathways

and leading to loss control over cell proliferation, differentiation and adhesion (54-56). Model for Bcr-Abl1 kinase activation suggested that the transforming activity of Bcr-Abl1 requires the coil-coil domain from the Bcr moiety, which mediates dimerization or tetramerization of Bcr-Abl1 (Fig 1.2) (57,58). Oligomerization of the kinases promote intermolecular autophosphorylation of the activation loop and other sites and lead to full catalytic activity (54,57). Disrupting coiled-coil domain oligomerization by mutation or using a peptide competitor reduces kinase and transforming activity, which support this activation model (Fig 1.2) (54,57,59,60).

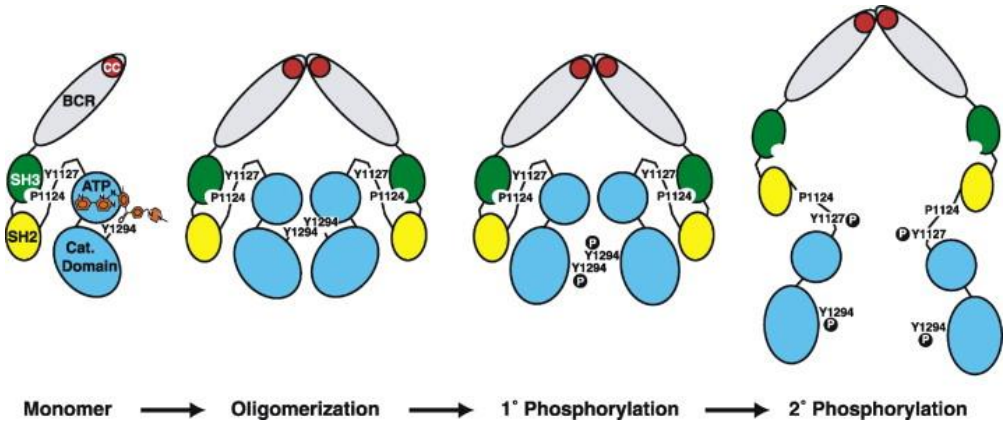


Figure 1.2 Model for Bcr-Abl Kinase Activation

Oligomerization of Bcr-Abl1 through the Bcr coil-coil domain promote kinase hyperactivation through intermolecular phosphorylation of key regulatory tyrosines. In addition, oligomerization and phosphorylation disrupt the auto-inhibitory interactions within the Abl N-terminal domains. Figure adapted from (57).

The development of small molecular inhibitors that target Bcr-Abl1 for the treatment of Chronic Myelogenous Leukemia (CML) was a major breakthrough that marked the era of targeted therapies. The discovery of Imatinib, also known as “Gleevec”, was widely regarded as the most successful cancer therapeutic ever developed. Although imatinib has proved to be remarkably successful for treating CML, many imatinib resistant mutations had been identified in patients. To overcome this disadvantage, several generations of ATP-competitive Abl kinase inhibitors including dasatinib, ponatinib, nilotinib and bosutinib were developed and approved by FDA (61).They were designed to bind both the inactive (nilotinib and ponatinib) and active (dasatinib and bosutinib) conformations of the Abl kinase activation loop. Furthermore, allosteric inhibitors including GNF-2 and GNF-5 were developed to target the myristoyl-binding pocket in the C-lobe of the Abl kinase domain in an attempt to bypass the T315I “gatekeeper” resistant mutation (62). A combination treatment of GNF-5 with imatinib or nilotinib was shown to prolong the survival in a Bcr-Abl1 T315I-induced leukemia mouse model (62). Therefore, the combine use of ATP-competitive and allosteric inhibitors represents an innovative and effective strategy to overcome resistance to either type of inhibitor alone. Besides their success in the clinical setting, these tyrosine kinase inhibitors were used in many co-crystal structures with Abl kinases, and provide great insight to the understanding of Abl kinase regulation (62-64) .

The "latch-clamp-switch" mechanism: Comparison between Abl and Src.

Similar to Src-family kinases, Abl family kinases are regulated via the "latch-clamp-switch" mechanism (Fig 1.3A) (54,65). In their natively inactive state, both Src and Abl kinase domains are held in a rigid conformation through intramolecular interaction with their respective SH3 and SH2 domain; The SH3 and SH2 domain acts as a "clamp" and form an inhibitory scaffold that fold along the back of the kinase domain, and this clamp is held together with the aid of molecular "latch" (Fig 1.3A) (27,54,65). Despite the high degree of conservation in the N-terminal domains of Abl and Src, there is significant difference on their detailed regulatory mechanism which contritbue to their distinct cellular function – the main difference being the "latch". In the case of Src, the latch is an intramolecular interaction betweeen the SH2 domain and phosphotyrosine (pY527) on the C-terminal tail (65). Phosphorylation of Src Y527 by other kinases inhibit Src kinase activity, while dephosphorylation of pY527 by phosphatase activate it (66,67). However, in the case of Abl family kinases, the N-terminal myristoyl group binding to the hydrophobic cavity of the kinase C-lobe serves as a latch by inducing a conformational change in the C-terminal kinase domain helix which allows the docking of SH2 domain on to the kinase C-terminal lobe (27,68). Stimulation of Abl and Src family kinases is initiated by releasing the latch and clamp, allowing the kinase domain to be flexible (Fig 1.3B). This process is usually achieved by engagement of the SH3 and SH2 domain with cellular binding partners (44,68,69). For full kinase activation, both Src and Abl fmialy kinases need to be phopshoryalted on several key tyrsoine resides

(45,46,65). Phosphorylation is believed act as a “switch” and promote the adoption of an active kinase state, preventing the return back to its inactive state (Fig 1.3B) (65).

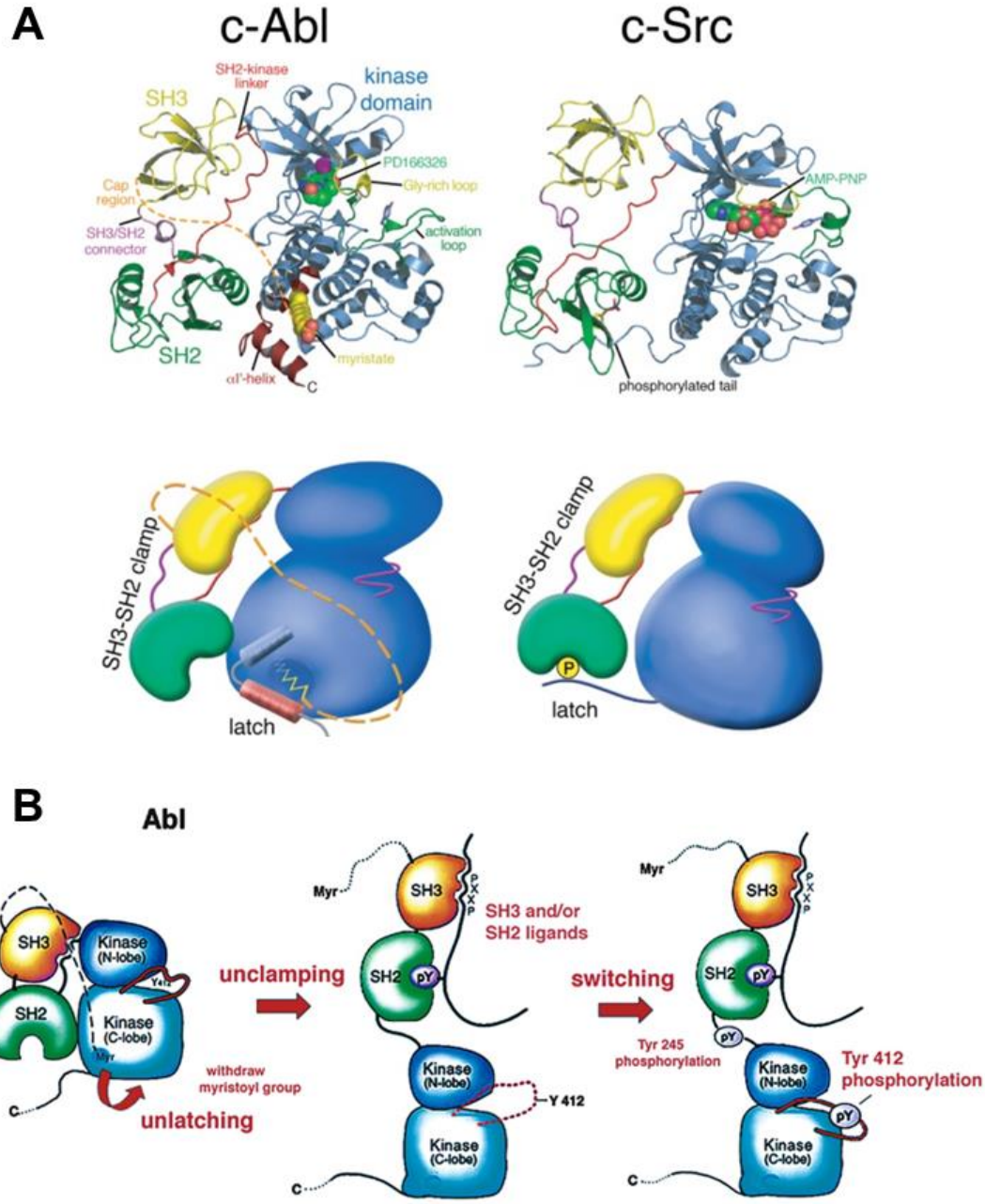


Figure 1.3 Abl and Src kinase are autoinhibited vis a “latch-clamp-switch” mechanism (Figure caption on next page)

Figure 1.3 Abl and Src kinase are autoinhibited vis a "latch-clamp-switch" mechanism

(A) Comparison of the "latch-clamp-switch" autoinhibitory structure between Src (PDB: 2SRC) and Abl1 (PDB: 1OPK). For both kinases, the SH3 and SH2 domain form the clamp that inhibit the kinase domain. For Src, intramolecular interaction between the SH2 domain and phosphotyrosine (pY527) on the C-terminal tail latches the clamp. For Abl, the N-terminal myristoyl group binding to the hydrophobic cavity of the kinase C-lobe serves as a latch by inducing a conformational change in the C-terminal kinase domain helix that allows the docking of SH2 domain on to the kinase C-terminal lobe. As for both kinases, tyrosine phosphorylation within the kinase domain serves to "switch on" the kinase activation loop. (B) Model of Abl family kinase activation propose that engagement of the SH3 and SH2 domains with cellular binding partners release the "clamp". Subsequent phosphorylation both at the Abl1/Abl2 SH2-kinase linker (Y245/Y272) and Abl1/Abl2 activation loop (Y412/Y439) act as a switch and promote the adoption of an active kinase state. Figure adapted from (54,65)

The Abl myristoylated N-cap is important for kinase regulation.

The Abl family kinases N-cap is about 80 amino acids in length and is myristoylated in the Abl1 1b (IV in mouse) and Abl2 1b isoform (Fig 1.1). The myristoyl group is a saturated long-chain fatty acid with a 14-carbon backbone. The crystal structure of the autoinhibited Abl1 core revealed that the N-cap myristoyl group buries in a deep hydrophobic pocket in the base of the kinase C-lobe with micromolar affinity (Fig 1.4A) (27,68). Myristoyl group binding induces a conformational change in the kinase domain αI helix, bending it 90° and allowing the SH2 domain to dock onto the kinase C-lobe (Fig1.4B) (27). Without the myristoyl group, the kinase αI helix is extended, causing a steric clash with the SH2 domain and disrupting the interaction (27). Interaction of the myristoylated N-cap with the kinase domain is important in maintaining Abl kinase in an autoinhibited conformation, as deletion or mutation of the myritoylation signal sequence leads to increased kinase and transforming activity (68,70). As aforementioned, small molecules GNF-2 and GNF-5 that bind to the myristoyl pocket act as allosteric inhibitors to regulate Abl kinase activity (62). Crystal structure of the modified N-Cap also reveal that there are multiple contact points between the N-Cap and the SH3/SH2 domains. Phosphorylated Ser69 can form hydrogen bond with Ser146 in the linker between SH3 and SH2, providing additional regulatory switch (71).

Although protein myristoylation is implicated in targeting proteins to the cell membrane, Abl N-cap myristoylation does not appear to play a major role in the localization of Abl kinases (68). Mutated non myristoylated Abl1 was not

differentially localized in cell compare to myristoylated Abl1(68). The cytoskeletal interaction domains in C terminal half of Abl kinases may be the major determinant of cellular localization as discussed before (Fig 1.1) (18,29,31).

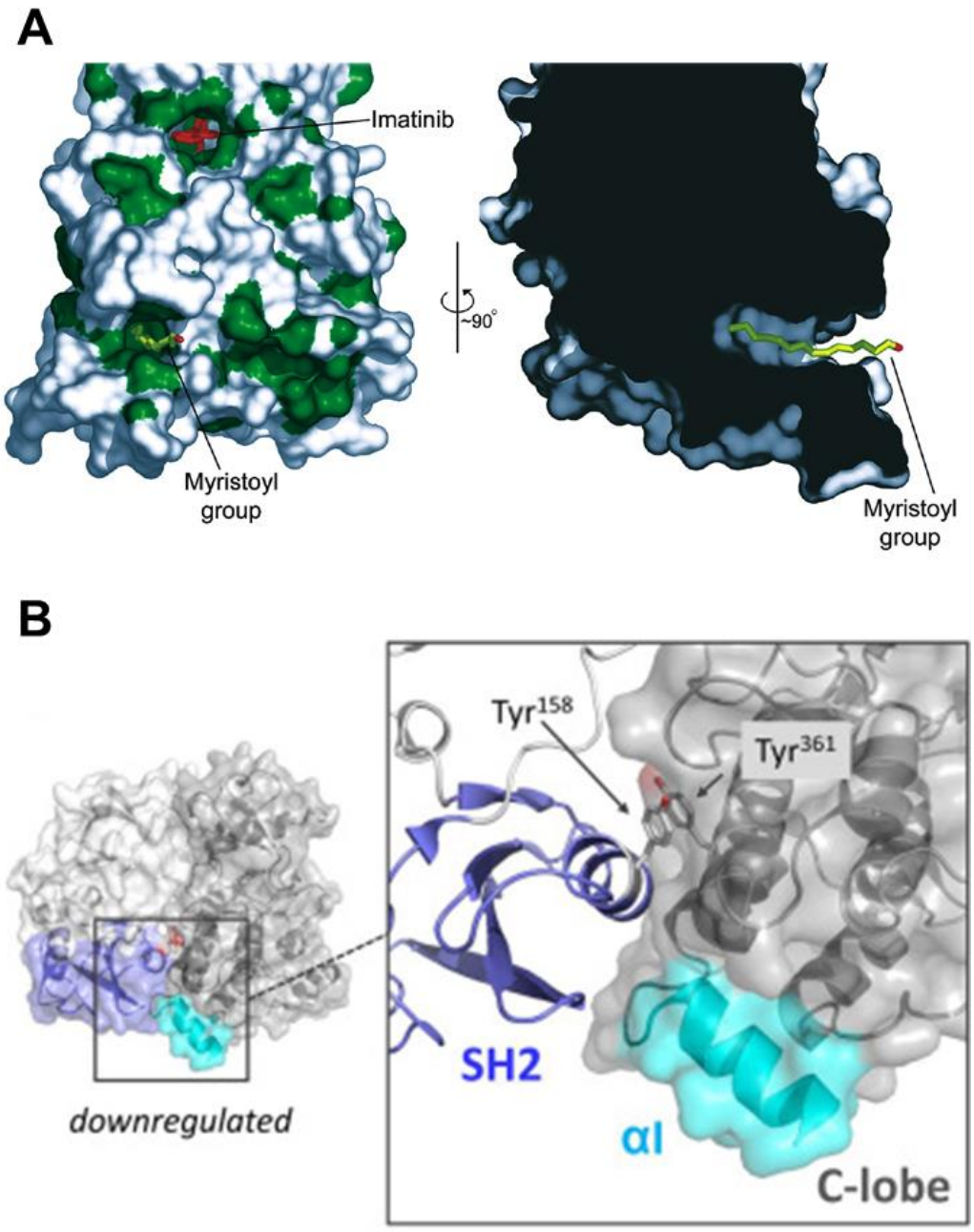


Figure 1.4 The Abl myristoyl group binds to the hydrophobic pocket in the Abl kinase domain to regulate its activity. (Figure caption on next page)

Figure 1.4 The Abl myristoyl group binds to the hydrophobic pocket in the Abl kinase domain to regulate its activity.

(A) (Left) Molecular surface of Abl1 kinase domain (PDB entry 1OPJ). Hydrophobic sidechains are colored as green. (right) Space fill model showing that the Abl myristoyl group binding in the hydrophobic pocket of the kinase domain.

(B) The interface of the SH2 domain and the kinase domain is highlighted in the autoinhibited structure of Abl1 (PDB entry 2FO0). The αI helix of the kinase domain (colored cyan) is rotated away from the SH2 domain, allowing SH2 (colored blue) to dock onto the kinase domain through pi-stacking interaction between Tyr158 and Tyr361. Figure adapted from (27,71,72)

The Abl SH3 domain, SH2-Kinase linker and the Kinase N-lobe form a sandwich like interaction.

Like other SH3 domain, the Abl SH3 domain binds to proline-rich peptide that form a poly proline type II (PPII) helix. In the auto-inhibited Abl kinases, the SH3 domain binds to the proline rich linker between the SH2 and kinase domains, which adopt a PPII helical conformation (Fig 1.5) (27,35,71,73). Mutations of the SH3 domain or the linker prolines perturbs this intramolecular interaction thereby activating Abl kinase activity (33,34,68). Direct competition with a proline-rich ligand or protein can also result in kinase activation (74,75). Interestingly, the second proline residue of the PXXP motif in the SH2-kinase linker is replaced by Tyr 245 in Abl1 (Tyr272 in Abl2). This tyrosine residue points away from the SH3 domain and packs into a hydrophobic crevice of the kinase N-lobe (27). Therefore, the SH2-kianse linker acts as a glue, sandwiched between the SH3 and kinase domain to mediate the clamping mechanism (27).

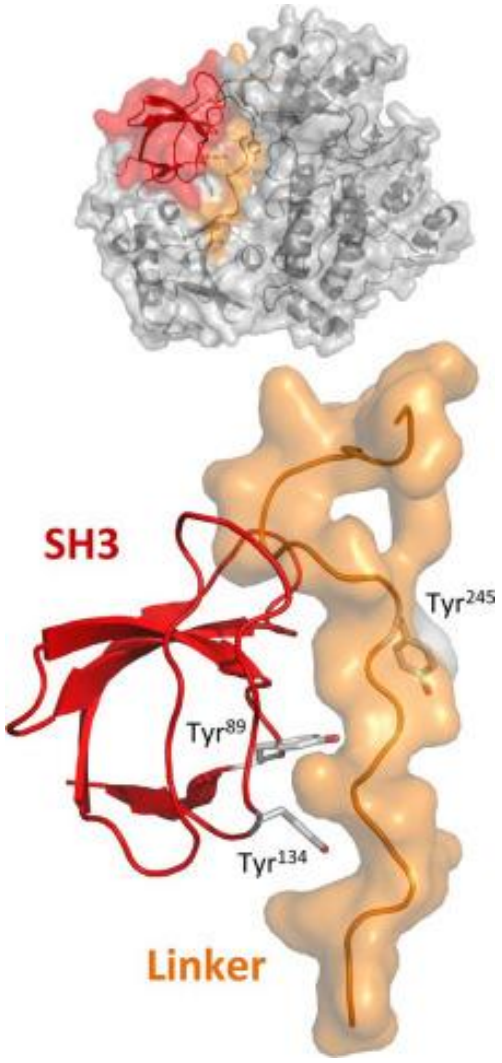


Figure 1.5 Abl SH3 domain interacts with the SH2-kinase linker

The position of the SH3 domain (red) and the SH2-kinase linker (orange) is highlighted in the autoinhibited structure of Abl1 (PDB entry 2FO0). The linker between SH2 and kinase domain form a PPII helix that interacts with the SH3 domain. Three regulatory tyrosine sites are also shown here. Figure adapted from (72)

The Abl SH2-kinase interaction changes dramatically between the inactive and active conformation.

As discussed above, the role of Abl SH2 domain in regulating Abl kinase activity differs from Src family kinase. The Src SH2 domain binds to the phosphorylated Tyr527 residue on the C-terminal tail, which serves as a "latch" in the autoinhibited conformation (Fig1.3) (65). In contrast, the phosphotyrosine-binding function of Abl SH2 domain does not contribute to the auto-inhibited conformation. Instead, it forms an extensive interaction interface with the Abl kinase C-lobe (27). The binding of the myristoyl group to kinase C-lobe hydrophobic pocket induces a conformational change of the αI helix to allow SH2 docking (27). Mutations that disrupt the SH2-kinase binding interface increase kinase activity (68). In the autoinhibited conformation, Abl SH2-kinase interface partially occludes access of phosphotyrosine ligand to the SH2 binding pocket, suggesting that the phosphotyrosine ligand or another Abl SH2 binding partner may break the SH2-kinase interaction hence activating kinase activity (27,44). A few structural studies have suggested that the Abl SH2 domain undergo significant rearrangement upon kinase activation (27,71). In the active state, Abl adopts an elongated configuration, where the SH2 domain no longer binds to the kinase C-lobe, but forms an extensive interface with the kinase N-lobe (Fig1.6) (71). Since the SH2 domain is on "top" of the kinase domain, this conformation is referred as the "top-hat" conformation (Fig1.6). Furthermore, the interaction between SH2 and the kinase N-lobe mediates allosteric activation of the kinase

domain (76). Mutations that disrupt the SH2/ kinase N-lobe interface impairs Abl kinase activity (77,78).

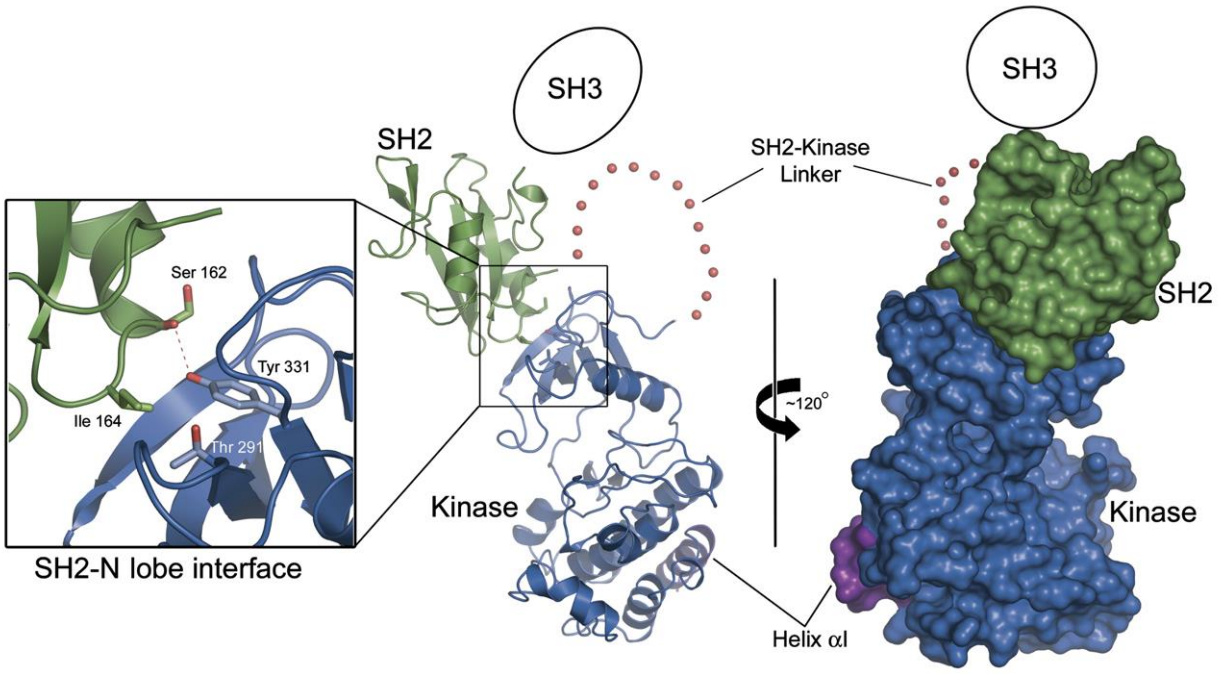


Figure 1.6 SH2-kinase interaction in active Abl1

The Abl1 SH2 domain interacts with the kinase domain N-terminal lobe to stabilize an active “top-hat” conformation. Key interaction resides are indicated. Figure adapted from (71).

Abl kinases are regulated by phosphorylation.

The auto-inhibited Abl kinases are regulated through a complex set of intramolecular interaction, and it is not phosphorylated on tyrosine residues (27,45,68,79). Disruption of the inhibitory interactions by mutagenesis result in higher Abl phosphotyrosine content, which positively correlates with increased kinase activity (34,45). Tyrosine phosphorylation in the kinase activation loop, the SH3 domain, the SH2-kinase linker, and elsewhere contributes to kinase activation, usually by disrupting regulatory intramolecular interactions. One example is the autophosphorylation of Abl1 Tyr245 (Tyr 272 in Abl2) in the SH2-kinase linker greatly increasing kinase activity, which results from disruption of the SH3/linker or the linker/kinase N-lobe interactions (45,46). Consistent with this, recent studies demonstrated that phosphorylation of Abl1 Tyr89 and Tyr134 (Tyr116 and Tyr161 in Abl2) prevents engagement of Abl SH3 domain with the SH2-kinase linker and are associated with enhanced Abl kinase activity (Fig 1.5) (80,81). Phosphorylation on the Abl kinase activation loop (Tyr412 in Abl1 and Tyr439 in Abl2) was proposed to stabilize a conformation that is more compatible with substrate biding and catalysis, thus increase its kinase activity (45,46). There are other Abl kinase phosphorylation sites identified using mass spectrometry (82-84). More studies are required to determine if these phosphorylation events have any role in regulating kinase activity or in specific cellular functions.

Pathways and receptors that activate Abl family kinases

Abl family kinases are activated by diverse upstream pathways and stimuli including receptor tyrosine kinases, adhesion receptors, immune cell receptors, DNA damage, cytokines and microbial invasion (Fig 1.7) (10,11,24,44,47,85-90) . This section will focus on the molecular mechanism by which different cell receptors signaling activate Abl kinases.

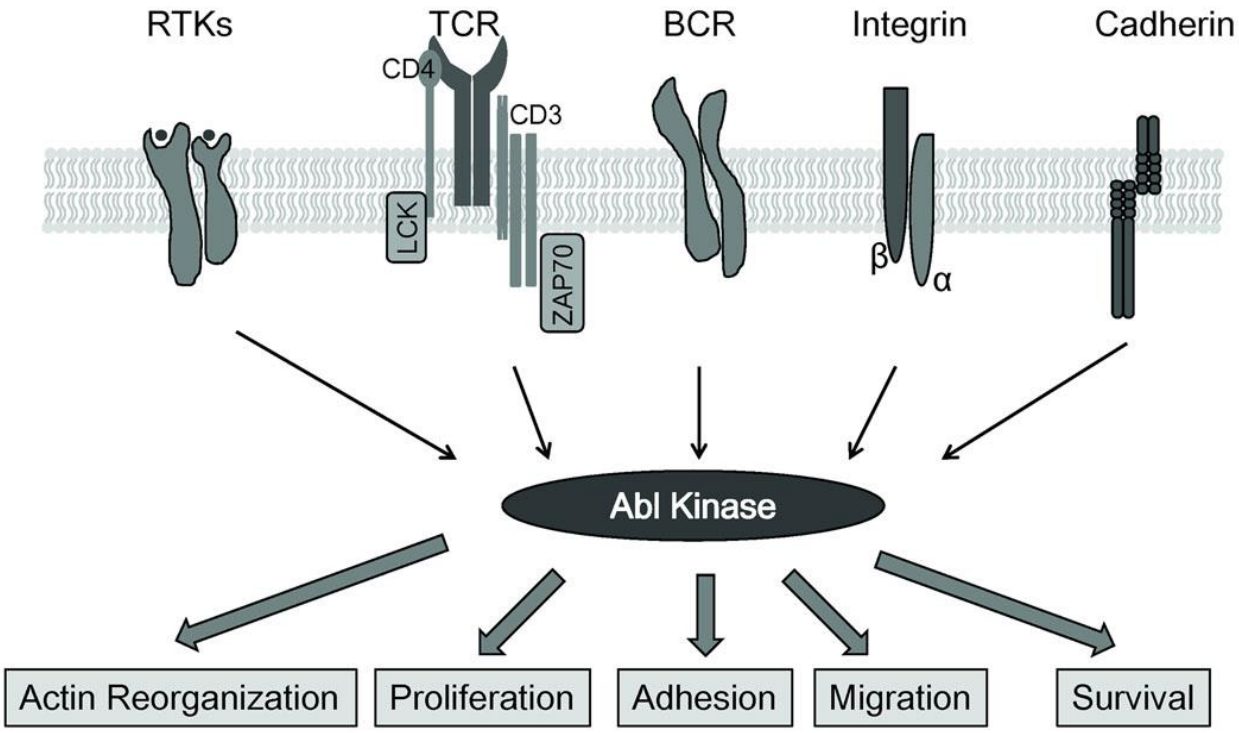


Figure 1.7 Multiple cell receptors activate Abl family kinases.

Abl family kinase are activated by diverse receptor singling including receptor tyrosine kinases, immune receptors and adhesion receptors. Abl kinases translate diverse extracellular signals to regulate cell proliferation, survival, migration and adhesion. Figure adapted from (90).

Activation of Abl kinases through integrin receptor signaling.

Integrins comprise of a large family of transmembrane adhesion receptors that provide a dynamic and bi-directional structural and signaling connection between the extracellular matrix and intracellular cytoskeleton (91). Early studies found that fibroblast plated on fibronectin or integrin cross-linking antibody have increased Abl1 kinase activity, as well as relocalization of Abl1 to early focal adhesions (92). Since then, our lab and others have further dissected the cellular functions by which integrin signaling promotes Abl kinase activation in a variety of cell contexts. Different studies show that integrin signaling induces Abl kinases dependent membrane protrusions which results from the phosphorylation of several Abl kinases substrate including p190RhoGAP and cortactin (18,31,50,88,93). Furthermore, laminin, an integrin ligand, acts through integrin-Abl1/Abl2-p190RhoGAp signaling cascade to regulate dendritic spine density and neurite branching (94-96). Our lab has recently demonstrated that Abl2 kinase domain directly interacts and phosphorylates integrin β1 cytoplasmic domain on Tyr-783. This phosphotyrosine then provide another binding interface for Abl2 SH2 domain, and these interfaces mediate integrin β1-Abl2 interactions in cells and enhance Abl2 kinase activity (Fig1.8) (44).

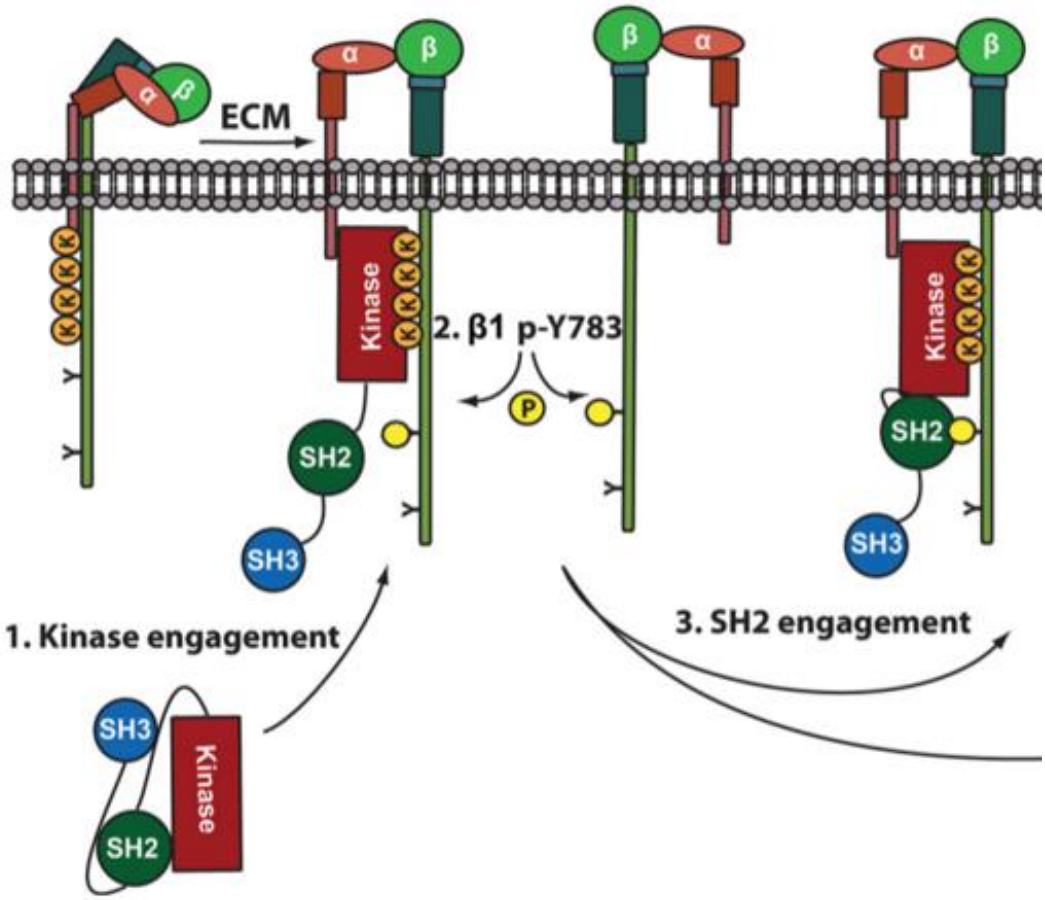


Figure 1.8 Model for Abl2 kinase activation by integrin β1

Abl2 kinase domain binds to the lysine rich region of integrin β1 cytoplasmic domain and phosphorylation Tyr783 residue. The phosphotyrosine provide additional binding interface for Abl2 SH2 domain. These indirect interaction interfaces may disrupt the autoinhibitory conformation of Abl2, leading to kinase activation. Figure adapted from (44)

Activation of Abl kinases through immune cell receptor signaling

The hypothesis that Abl family kinase might have a role in immune B and T cell receptors signaling came from early studies suggesting that mice lacking Abl1 or Abl C-terminal half exhibited immune deficit phenotypes *i.e.* increased susceptibility to infection, and harboring smaller thymus and spleen sizes (97,98). Abl1 deficient mice also showed decreased B and T cell numbers (98). Later studies show that Abl1 kinase activity and protein level is increased upon B cell receptor (BCR) activation in B-cell lines. This study also shows that BCR coreceptor CD19, as a substrate of Abl1 kinase, was phosphorylated after B-cell activation, resulting in the recruitment of Abl1 and other SH2 containing proteins (99). To investigate the potential role of Abl kinases in T cell signaling, a study showed that endogenous Abl kinases are activated following T cell receptor (TCR) stimulation, and the activation partially requires Src family kinase Lck (14). This study further identified ZAP-70 and LAT as substrates of Abl kinases; pharmaceutical inhibition of Abl kinases activity with STI571 or reduced Abl kinases expression results in decreased phosphorylation of ZAP-70 and LAT. Conditional knock out of Abl1 and Abl2 in primary T cells show impaired TCR dependent activation of ZAP-70, LAT, Jnk, Shc and PLCγ1 (14). These studies showed that Abl kinases acts downstream of immune B and T cell receptors signaling, and are required for linking pre-receptor stimulation to activation/phosphorylation of downstream signaling molecules (14,99). However, the molecular mechanism by which Abl kinases are activated by immune cell receptors signaling remains unclear. The specific role of Abl kinases in

phosphorylating multiple signaling molecules under immune receptor signaling is yet to be determined.

Activation of Abl kinases through growth factor receptors

Early studies suggest that growth factor including platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) stimulation promote Abl family kinases activation (47,85,86). Both Src family kinases and phospholipase C-γ1 (PLC-γ1) are required for the full activation of Abl kinases by growth factor receptors (47,85,86). Src family kinases phosphorylate Abl kinases on the activation loop, and PDGF activation of Abl kinases is reduced in $src^{-/-}yes^{-/-}fyn^{-/-}$ cell lacking Src kinase activity (45,46,85). In addition, PLC- γ1 plays an important role in this pathway by hydrolyzing PI(4,5)P2, which is identified as an Abl kinases inhibitor (47,86). Our lab and others showed that Abl kinases mediate the biological effect of PDGF-induced dorsal membrane ruffles, cell proliferation and chemotaxis (47,48,85-87). EGFR and PDGFR also promote Abl kinase activity in different cancer cells (4,5,7,100). PDGFR signaling through Abl1 is up regulated during the development of resistance to aromatase inhibitor treatment in breast cancer (101).

Thesis project aims and my contributions

1. To determine the molecular mechanism by which Platelet Derived growth factor receptor beta (PDGFRβ) activates Abl2.

This project started from an interesting preliminary discovery that PDGFRβ interacts with Abl2 *in vitro* with sub-micromolar affinity using microscale thermophoresis. With previous studies showing that PDGF stimulation promote Abl2 kinase activity, I continued with this initial finding and determined the molecular mechanism by which PDGFRβ interacts with, phosphorylates, and activates Abl2 kinase (details described in Chapter 2). I found that phospho-Y771 in PDGFRβ is required to interact with the Abl2 SH2 domain both *in vitro* and in cells. I demonstrated that PDGFRβ directly phosphorylates Abl2 on multiple novel sites within the SH3/SH2-kinase linker interface, which normally acts to keep the Abl2 in the auto-inhibited conformation. (Phosphopeptide mapping with mass spectrometry is performed in collaboration with Dr.Yougjoo Kim in Dr. Karen Anderson's lab and Dr. Hanzhi Wu in Dr. Cristina Furdui's lab). I showed that PDGFRβ phosphorylation on Abl2 results in Abl2 kinase activation both in vitro and in cells. Mutation of the PDGFRβ phosphorylation sites on Abl2 to nonphosphorylatable residues abrogate PDGFRβ-mediated activation of Abl2 kinase activity. Together, these findings provide a molecular mechanism to understand how receptor tyrosine kinases active Abl family kinase through different phosphorylation events.

2. To determine whether PDGFR regulates integrin activation through Abl2 signaling.

This project started from an interesting discovery that PDGF stimulation promotes Abl2-integrinβ1 interaction by a former graduate student, Adam Simpson. He also found that Abl2 directly binds and phosphorylates integrin β1. Interestingly, Abl2's binding sites on integrin β1 overlap with those of talin, which is an important integrin intracellular activator. I continued with this initial finding and later discovered that PDGF stimulation also promotes Abl2 phosphorylation on integrin β1 Y783, which had been shown to block talin's binding on integrin β1(102,103). Therefore, I hypothesized that PDGFR signaling may regulate talin-mediated integrin activation through either direct competition or phosphorylation (details described in Chapter 3). I found that Abl2 competes with talin for integrin β1 binding in pull down assays. In collaboration with Dr. Maddy Parsons using Förster resonance energy transfer by fluorescence lifetime imaging microscopy (FLET-FLIM), we found that Abl2 expression led to a strong decrease in interaction between integrin β1 and talin in response to PDGF stimulation in cell, and this effect is greatly reduced in Abl2 knock down cell. In collaboration with Daniel Iwamoto in Dr. David Calderwood's lab, we used a Fluorescence-activated Cell Sorting (FACS) based assay to measure integrin activation. Unfortunately, results consistently show that there is no significant effect on integrin activation after PDGF stimulation or Abl2 overexpression. Nonetheless, these studies provide a basis for future studies in dissecting the molecular mechanism by which PDGFR signaling regulate cell adhesion, migration and invasion through Abl2/talin/integrin interactions.

CHAPTER 2- PLATELET-DERIVED GROWTH FACTOR RECEPTOR BETA ACTIVATES ABL2 THROUGH DIRECT BINDING AND PHOSPHORYLATION.

Abstract

The catalytic activity of Abl family kinases is tightly regulated in cells by a complex set of intermolecular and intramolecular interactions and post-translational modifications. Abl kinases are activated by diverse cellular stimuli, one of them is receptor tyrosine kinase signaling. PDGFRβ is identified as a potent activator of Abl family kinases, and these kinases mediate the biological effects of PDGF. However, molecular mechanism by which PDGFRβ engages and activates Abl family kinases is not known. Here, we report the molecular mechanism by which PDGFRβ interacts, phosphorylates and activates Abl2 kinase. We found that PDGFRβ binds and phosphorylates Abl2 both in vitro and in cells. We also identified several novel PDGFRβ phosphorylation sites on Abl2, including Y116, Y139 and Y161 on the SH3 domain, Y299, Y303 and Y310 on the kinase domain. Of notable interest, Y116, Y161, Y272 and Y310 are all located near the SH3/SH2-kinase linker interface, which help maintain Abl family kinases in an auto-inhibited conformation. Mutation of these four tyrosine (Y116, Y161, Y272 and Y310) to phenylalanine abrogated PDGFRβ-mediated activation of Abl2 kinase activity. These findings provide a mechanism to understand how receptor tyrosine kinases activate Abl family kinases, and how Abl kinase are precisely regulated through different phosphorylation events.

Introduction

Abl family nonreceptor tyrosine kinases, comprised of Abl1 and Abl2 in vertebrates, translate signals from growth factors and adhesion receptors to regulate cytoskeleton organization and remodeling, which is essential to many cellular processes including cell morphogenesis, adhesion and migration (1-9). The catalytic activity of Abl family kinases is important in promoting actin-based cell edge protrusions, facilitating endocytosis and phagocytosis, mediating DNA damage responses, and regulating cell survival and proliferation in a variety of cell contexts. These processes play essential roles in the development and function of the cardiovascular, brain, and immune systems, among others (6,8,10-19).

The catalytic activity of Abl family kinases is tightly regulated, and inappropriate kinase regulation drives leukemia development and promotes solid tumor progression (18,20-24). The kinase activities of Abl1 and Abl2 are regulated by a complex set of intermolecular and intramolecular interactions and post-translational modifications (25-29). Non-activated Abl kinases are kept inactive via an autoinhibitory mechanism, in which the kinase domain is held in a rigid conformation through intramolecular interactions with the SH3 and SH2 domains (27-30). Models for kinase activation proposed that engagement of SH3 and SH2 domains with cellular binding partners relieves this inhibition. Subsequent tyrosine phosphorylation events promote adoption of an active conformation and prevent returning back to the inactive conformation (9,25,26,29,31). Endogenous Abl kinases are activated by diverse stimuli

including growth factors, cytokines, DNA damage and adhesion receptors (1-4,7,9,31,32).

Abl family kinases are activated downstream of receptor tyrosine kinases in fibroblast and cancer cells, including the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (1,2,7,18,21,23). The PDGFRβ is an especially potent activator of Abl family kinases, and Abl kinases mediate the biological effects of PDGF including PDGF-induced dorsal membrane ruffles, cell proliferation and chemotaxis (1-3,7,33). PDGFR signaling through Abl1 is upregulated during the development of resistance to aromatase inhibitor treatment in breast cancer (34). Previous work showed that the PDGFRβ binds Abl kinases and this is associated with increased Abl kinase activation (1-3,7), but the molecular mechanism by which PDGFRβ engages and activates Abl family kinases is not known.

Here we report the molecular mechanism by which PDGFRβ interacts with, phosphorylates and activates Abl2 kinase. We found that PDGFRβ binds and phosphorylates Abl2 both in vitro and in cells. We also identified several novel tyrosine (Y) phosphorylation sites on Abl2 including Y116, Y139 and Y161 on the SH3 domain and Y299, Y303 and Y310 on the kinase domain. Of notable interest, Y116, Y161, Y272 and Y310 are all located near the SH3/SH2-kinase linker interface, which is crucial for keeping Abl2 in an auto-inhibited conformation. Mutation of Y116, Y161, Y272 and Y310 to phenylalanine abrogated PDGFRβ mediated activation on Abl2. These findings provide a

mechanism to understand how Abl family kinases are regulated by receptor tyrosine kinases through different phosphorylation events.

Results

The Abl2 SH2 domain binds to phosphotyrosine 771 in PDGFRβ.

Previous work demonstrated that Abl2 co-immunoprecipitates with PDGFRβ from cell lysates (47), but whether the PDGFRβ binds Abl2 directly or which interfaces mediate this interaction are not known. Upon activation by PDGF binding, the PDGFRβ cytoplasmic domain undergoes tyrosine autophosphorylation at multiple sites, which recruit key adaptor and signaling proteins. We hypothesized that Abl2 SH2 domain directly binds one or more of these phosphotyrosines.

PDGFRβ was expressed in HEK293 cells and activated by stimulation with PDGF-BB (PDGF). Following stimulation, we found that PDGFRβ could be retained on Abl2 SH2-domain containing agarose beads, but not to beads containing the Abl2 SH2 R198K point mutation that disrupts SH2 binding to phosphotyrosine-containing binding partners (18,104) (Fig 2.1B). In parallel, the kinase-inactive PDGFRβ point mutant (K634R) did not undergo PDGF-stimulated autophosphorylation and was not retained on Abl2 SH2 beads (Fig 2.1A, B). These data indicated that autophosphorylated PDGFRβ in cell lysates can bind the Abl2 SH2 domain.

PDGFRβ is phosphorylated at multiple sites in cells, some or all of which could be binding interfaces for the Abl2 SH2 domain, but previous studies have not resolved which phosphotyrosine (pY) residue of PDGFRβ serve as binding sites for the Abl2 SH2 domain. To address this, we mutated specific tyrosine residues in the PDGFRβ cytoplasmic domain to phenylalanine and expressed the mutants in HEK293 cells. All mutants were expressed at similar levels and underwent significant tyrosine phosphorylation following PDGF-BB treatment, but binding of the PDGFRβ Y771F mutant to Abl2 SH2 domain beads was reduced by 90% relative to WT PDGFRβ or any of the other Y to F single substitution mutants of PDGFRβ (Fig 2.1C, D). Binding of PDGFRβ Y751F was also reduced, but only by 10% relative to wild type controls. (Fig 2.1C, D) These data suggest that phospho-Y771 in PDGFRβ is required to interact with the Abl2 SH2 domain.

Before I perform the mutagenesis study to determine the binding interface between Abl2 and PDGFRβ, we also looked at SH2 screen publications and try to find a clue about potential binding interfaces with the help of Dr. Kazuya Machida. Even though most of studies have not determined or detected PDGFRβ/Abl2 interactions, these studies provide some insight (41,105). For example, a florescence polarization based high throughput screen show some consensus residues for known receptors that interact with Abl2 (Fig 2.2) (105). SH2 domain bind to phosphotyrosine-containing peptides with sequence specificity. Different SH2 domains have different preferences regarding the three to six amino acid residues downstream from the phosphorylated tyrosine (38,39).

Interestingly, the binding motif for PDGFRβ pY771 YMAP is similar to some known receptors that binds to Abl2. M in the first residue position and P in the third residue poison after the phosphotyrosine appear in a lot of Abl2 SH2 binders (Fig 2.2).

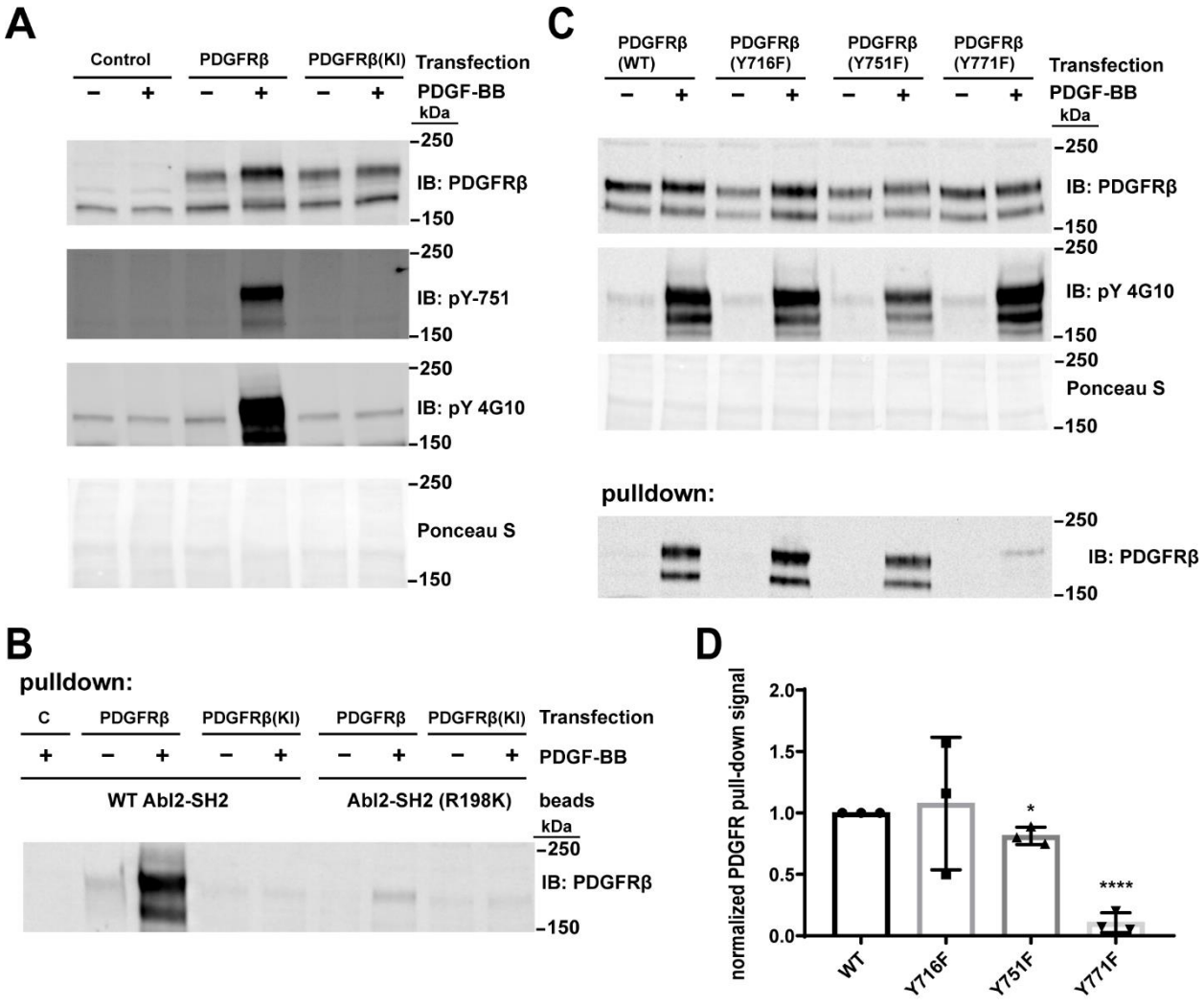


Figure 2.1 The Abl2 SH2 domain binds phosphorylated PDGFRβ. (Figure caption on next page)

Figure 2.1 The Abl2 SH2 domain binds phosphorylated PDGFRβ.

(A) HEK293 cells either untransfected (control) or transfected with PDGFRβ or kinase inactive (KI) PDGFRβ were serum starved overnight and treated with 100 ng/ml PDGF-BB for 10 minutes. 40 µg of lysate were immunoblotted with antibodies to PDGFRβ, phosphotyrosine-751 in PDGFRβ, or phosphotyrosine (4G10). Bottom panel shows the Ponceau S-stained blots as loading controls. Molecular weight markers are indicated. Significant tyrosine phosphorylation is shown by western blotting using both nonspecific phospho-tyrosine antibody and a specific phosphor-Y751 PDGFR antibody. (B) Agarose beads covalently coupled to WT Abl2 SH2 domain and phosphotyrosine-binding defective (R198K) Abl2 SH2 mutant were incubated with 500 µg HEK293 cell lysates expressing WT and KI PDGFRβ that were mock treated or stimulated with PDGF-BB after overnight serum starvation as in (A). WT Abl2 SH2 domain pull down PDGFRβ that had been stimulated with PDGF-BB. (C) (top 3 panels) Wild type and tyrosine (Y) to phenylalanine (F) PDGFRβ mutants (Y716F, Y751F and Y771F) were transiently expressed in HEK293 cells and stimulated as in (A). 40 µg of cell lysate was immunoblotted for PDGFRβ or phospho-tyrosine, while bottom panel shows Ponceau S staining as control. (bottom panel) 500 µg of the indicated lysates were incubated with beads coupled to the Abl2 SH2 domain and the bound material was immunoblotted for PDGFRβ. (D) Quantification of PDGFR pull down by Abl2 SH2 domain.

A

Protein	-3	-2	-1	0	1	2	3	4
ABL2	X	P,L,V	P,M,L	Y	E,M,D,S,A	M,V,E,I	P,T,V,M	M,L,V
ABL1	X	P	P	Y	E,D,M,V	M,V,N	V,L,P,I,A,T	M,G

B

Receptor	pTyr site	Sequence	Motif	Protein	Mean KD (μM)
AR	267	GDCMYAPLLGVPd	YAPL	ABL1	2.13
AR	553	PIDYYFPPQKTCd	YFPP	ABL1	9.44
ErbB2	1127	ETDGYVAPLTCSd	YVAP	ABL1	5.35
ErbB3	1307	PHVHYARLKTLR	YARL	ABL1	8.91
ErbB4	1150	DEEGYMTPMRDK	YMTP	ABL1	8.75
KIT	721	STNEYMDMKPGVd	YMDM	ABL1	8.36
KIT	672	VITEYCCYGDLLd	YCCY	ABL1	9.82
AR	553	PIDYYFPPQKTCd	YFPP	ABL2	11.30
ErbB2	685	KIRKYTMRRLLQd	YTMR	ABL2	11.39
ErbB2	1139	PQPEYVNQPDVRd	YVNQ	ABL2	18.43
ErbB3	1262	EDYEYMNRQRDGd	YMNR	ABL2	6.22
ErbB3	1307	PHVHYARLKTLR	YARL	ABL2	7.37
ErbB3	1224	LGYEYMDVGSDLd	YMDV	ABL2	8.27
ErbB4	1150	DEEGYMTPMRDK	YMTP	ABL2	10.40
ErbB4	1242	DNPDYWNHSLPPd	YWNH	ABL2	10.84
ErbB4	906	DVWSYGVTIWELd	YGVT	ABL2	12.08
KIT	936	TNHIYSNLANCSd	YSNL	ABL2	3.48
KIT	570	NNYVYIDPTQLPd	YIDP	ABL2	14.77
MET	1313	PDPLYEVMLKCWd	YEVM	ABL2	9.49

	pTyr site	Sequence	Motif
PDGFRb	562	QKKPRYEIRWK	YEIR
PDGFRb	579/581	SDGHEYIYVDPMQ	YIYV
PDGFRb	716	PSAELYSNALP	YSNA
PDGFRb	740	ESDGGYMDMSK	YMDM
PDGFRb	763	KGDVKYADIES	YADI
PDGFRb	771	IESSNYMAPYD	YMAP
PDGFRb	775	NYMAPYDNYVP	YDNY
PDGFRb	778	APYDNYVPSAP	YVPS
PDGFRb	857	MRDSNYISKGS	YISK
PDGFRb	1009	TSSVLYTAVQP	YTAV
PDGFRb	1021	EGDNDYIIPLP	YIIP

Figure 2.2 Summary of receptors that binds to Abl2 SH2 domain show consensus residues. (A) Prediction of Abl kinases SH2 binder's consensus is shown. (B) Summary of the binding sequence for other receptors that interacts

with Abl2 SH2 domain. Similar sites in PDGFR are highlighted. Data summarized from (105).

We next used purified recombinant Abl2 SH2 domain and PDGFRβ cytoplasmic domain (CD) to test whether the proteins interact directly and to measure the affinity and specificity of this interaction. The PDGFRβ CD was comprised of the residues spanning from C-terminal end of the transmembrane region to the C-terminus of the protein (554M-1106L). We purified 6XHis-tagged PDGFRβ CD following baculovirus-mediated expression in insect cells, fully dephosphorylated it in vitro using phage lambda phosphatase, and re-purified the dephosphorylated PDGFRβ CD (Fig 2.3A). We incubated PDGFRβ CD in the presence of saturating Mg^{2+} and ATP for 2 hours to enable it to autophosphorylate to completion (Fig 2.3A). Purified autophosphorylated PDGFRβ CD bound to the Abl2 SH2 domain beads with sub-micromolar affinity (K_d= 0.26 $\pm$ 0.07 μM), while the non-phosphorylated PDGFRβ CD only exhibited weak background binding (Fig 2.3B). As in experiments using cell-derived PDGFRβ (Fig 2.3B), the binding defective Abl2 SH2 domain R198K mutant completely abolished binding to auto-phosphorylated PDGFRβ CD (Fig 2.3C). While the PDGFRβ CD Y771F mutant was able to autophosphorylate in vitro, it did not bind significantly to Abl2 SH2 domain-containing beads (Fig 2.3A, D, E). Together, our data indicate that the

Abl2 SH2 domain binds directly to phosphorylated Y771 interface on the PDGFR cytoplasmic domain.

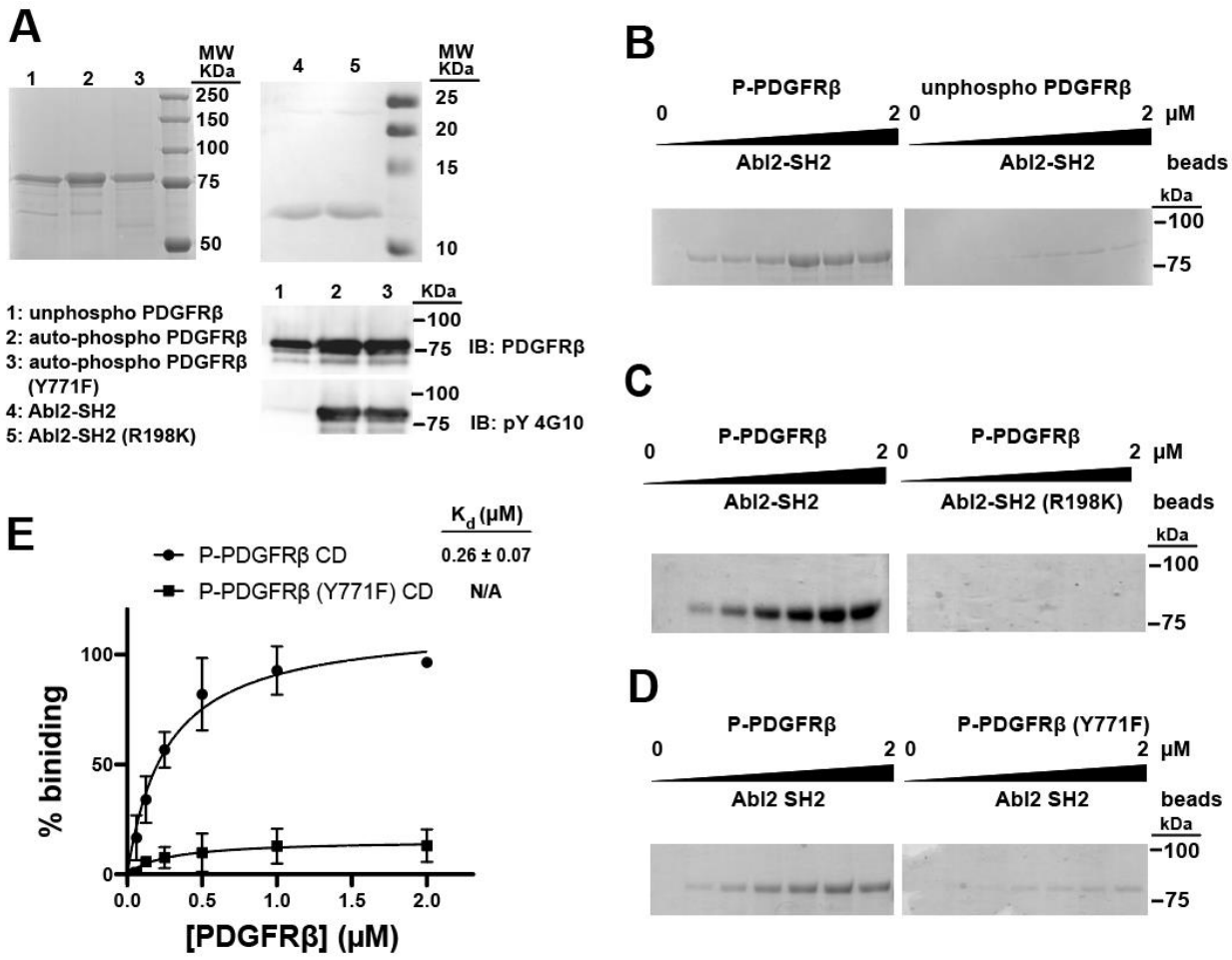


Figure 2.3 Abl2 SH2 directly binds phosphorylated PDGFRβ cytoplasmic domain in vitro. (Figure caption on next page)

Figure 2.3 Abl2 SH2 directly binds phosphorylated PDGFRβ cytoplasmic domain in vitro.

(A) Coomassie Blue-stained gel showing the purity of recombinant purified proteins used in this figure. 50 ng of recombinant PDGFRβ CD were immunoblotted with antibodies to PDGFRβ and phosphotyrosine (4G10). (B-E) The concentration dependence of auto-phosphorylated PDGFRβ CD, non-phosphorylated PDGFRβ CD (B), and auto-phosphorylated PDGFRβ CD (Y771F) (D) binding to Abl2 SH2 domain and phosphotyrosine-binding defective (R198K) Abl2 SH2 mutant (C) were measured. An increasing concentration of PDGFRβ CD from 0-2 μM in binding reaction were pulled down by agarose beads covalently coupled to Abl2 SH2 at a final concentration of 1 μM. Pulldown products were separated by SDS PAGE, and gel bands were resolved with Coomassie blue stain and densities were quantified using ImageJ. One-site specific binding isotherms fit using ImageJ was set to binding experiments. The K_d value for phosphorylated PDGFR CD and Abl2 SH2 domain is 0.26 ± 0.07 μM. Error bars represent S.E. from n=3.

PDGFRβ directly phosphorylates the Abl2 N-terminal half on multiple novel sites.

Abl2 kinase activity is activated by phosphorylation (46). We used an in vitro kinase assay to measure whether purified recombinant PDGFRβ CD phosphorylates Abl2. We expressed MBP-Abl2 full length (encompassing the first common exon to C-terminus), MBP-Abl2 C-terminus (residues 557-1182 around 120 Kda) and a 6XHis-tagged Abl2 N-terminus (residues 74-557) in insect cells and purified them (Fig 2.4A). The Abl2 kinase domain-containing constructs carried two inactivating mutations (D307N, K317M) in the kinase domain, which eliminates possible Abl2 autophosphorylation. In the presence of Mg^{2+} and ATP, 5 nM of recombinant PDGFRβ CD directly phosphorylated full length Abl2 and the Abl2 N-terminus, but only very weakly the Abl2 C-terminus (Fig 2.4B). We next investigated whether PDGFR/Abl2 direct interaction is required for PDGFR to phosphorylate Abl2. PDGFRβ CD phosphorylation of the Abl2 N-terminus (R198K) SH2 domain mutant, defective in binding, was greatly reduced relative to WT Abl2 N-terminus (Fig 2.4C). These results suggest that SH2 domain-mediated Abl2 recruitment to PDGFR is required for its phosphorylation.

Phosphorylation of Abl2 at Y272 in the SH2 domain-kinase linker and Y439 in the kinase activation loop can activate its kinase activity and Abl1 is similarly activated via phosphorylation of those homologous sites (45,46). We mutated these sites in the Abl2 N-terminus construct to test how this impacts phosphorylation by the PDGFRβ CD. Unexpectedly, we found that the PDGFRβ

CD could still phosphorylate Abl2 N-terminus Y272F/Y439F mutant with a similar k_{cat} (6.8 min^{-1} vs. 7.3 min^{-1}) and K_M (1.6 µM vs. 2.8 µM) as compared to WT, suggesting that the PDGFRβ CD phosphorylates one or more novel sites in Abl2 (Fig 2.4D).

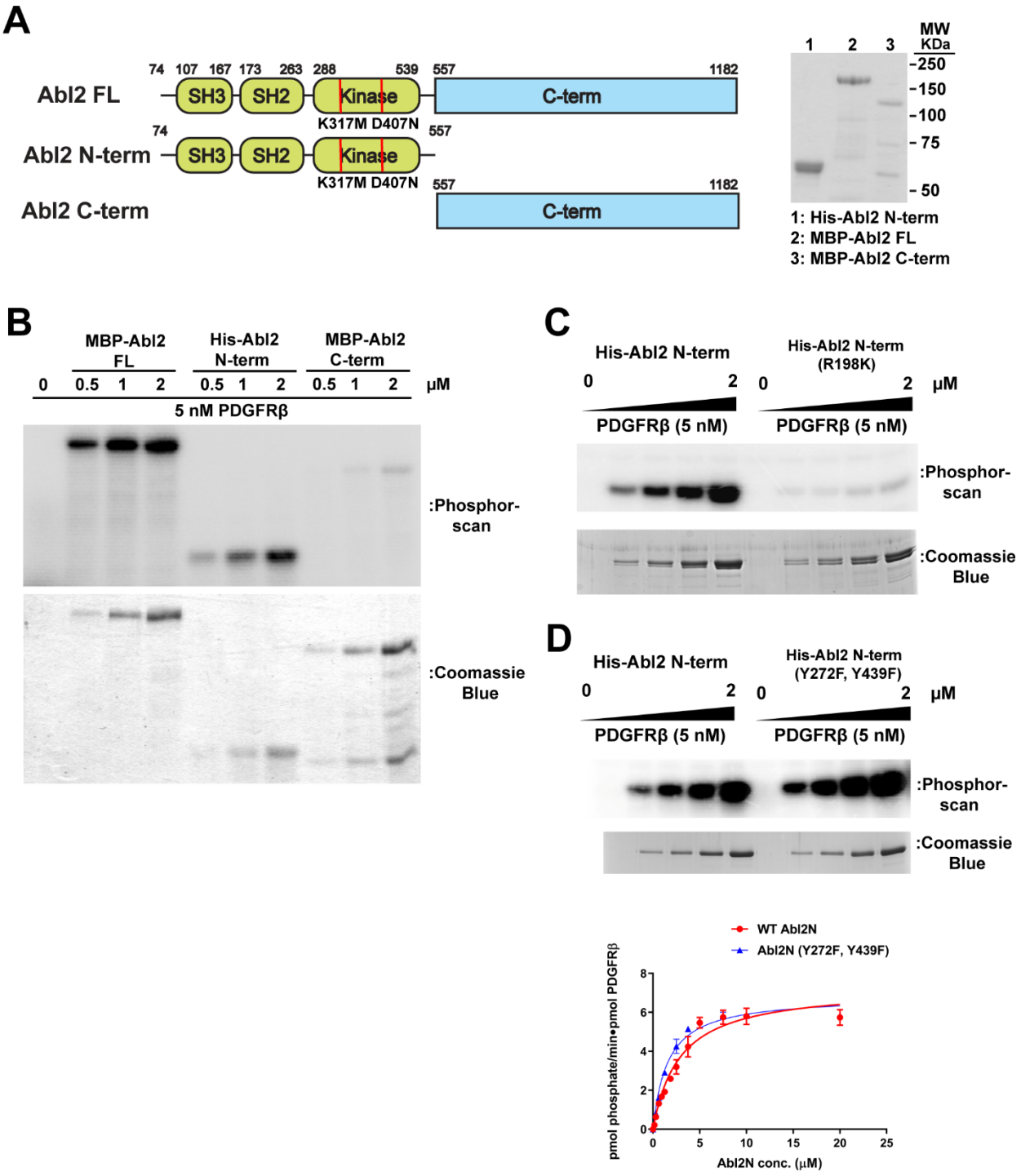


Figure 2.4 PDGFRβ phosphorylates the Abl2 N-terminal half.

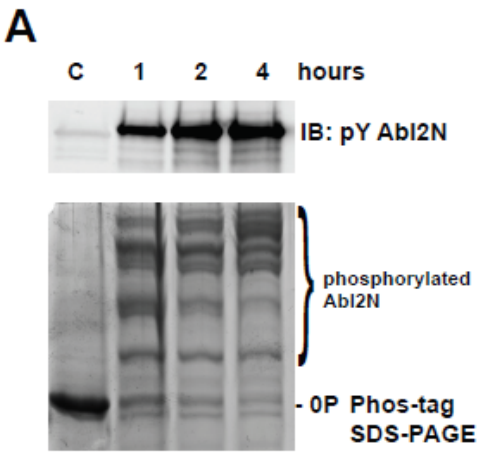
(Figure caption on next page)

Figure 2.4 PDGFRβ phosphorylates the Abl2 N-terminal half.

(A) Domain architecture and purified recombinant protein of Abl2 and Abl2 N- and C-terminal halves. Purified proteins were separated by SDS-PAGE and visualized by Coomassie Blue staining. The Abl2 kinase domain-containing constructs carried two inactivating mutations (D307N, K317M, indicated red lines) in the kinase domain to eliminate possible Abl2 autophosphorylation. (B) Radioactive ATP Kinase assays were performed by preincubating 5 nM PDGFRβ CD and 0-2 µM Abl2 constructs for 5 min at 32 °C before initiating reactions with 5 µM ATP and 0.75 µCi of [γ-^{32}P] ATP for 10 min before terminating with 1X LSB, running on gels, and exposing to a phosphor imaging screen. A parallel assay was performed without [γ-^{32}P] ATP addition, separated by SDS-PAGE and visualized with Coomassie Blue. (C) Radioactive ATP Kinase assays testing ability of PDGFRβ CD to phosphorylate Abl2 N-term and a phosphotyrosine-binding defective (R198K) Abl2 N-term mutant with PDGFRβ CD as kinase. A parallel SDS PAGE visualized with Coomassie Blue is performed. (D) (top panel) Radioactive ATP Kinase assays of WT Abl2 N-term and Abl2 N-term (Y272F Y439F) mutant phosphorylated by PDGFRβ CD as kinase. (bottom panel) 0-20µM WT Abl2 N-term and mutant were preincubated incubated with 0.1 nM of PDGFRβ CD in kinase assay conditions described in (B). Reactions were quenched with 1X LSB after 10 mins, boiled, and separated on 10% SDS PAGE and protein bands were stained with Blue Silver G-250 Coomassie to visualize Abl2 N-term protein bands. Bands were cut out and scintillation counted. Counts

per minute were converted and fit to Michaelis-Menten equation in GraphPad to obtain kinetic parameters. Error bars represent S.E. from n = 3.

In order to identify the novel phosphorylation sites, we performed phosphopeptide mapping with mass spectrometry (MS). Abl2N purified from insect cells was treated with a mix of Lambda protein phosphatase and YopH for dephosphorylation. Abl2N phosphorylated by PDGFRβ CD was monitored for phosphorylation status at different time points. Samples immunoblotted for phosphotyrosine show phosphorylation intensity saturates at 2 hours reaction time (Fig 2.5A, top panel). Phos-tag SDS-PAGE successfully separated unphosphorylated Abl2N and resolved multiple phosphorylated Abl2N species in the PDGFRβ CD-treated samples. As kinase reaction time increases, the intensity increases for higher phosphorylated states of Abl2N, while decreases for lower phosphorylated sates and non-phosphorylated Abl2N (Fig 2.5A lower panel). The phosphorylated Abl2N was used to perform phosphopeptide mapping by MS to locate potential new phosphotyrosine residues. MS analysis identified 7 tyrosine phosphorylation sites (Fig 2.5B), including phospho-Y439 which was previously identified as a Src family kinase-mediated phosphorylation site (46) (Fig 2.5C), and several novel sites including phospho-Y161(Fig 2.5D).



B

pY identified	pY location
Y139	SH3 domain
Y161	SH3 domain
Y272	SH2-kinase linker
Y299	kinase domain
Y303	kinase domain
Y310	kinase domain
Y439	kinase domain

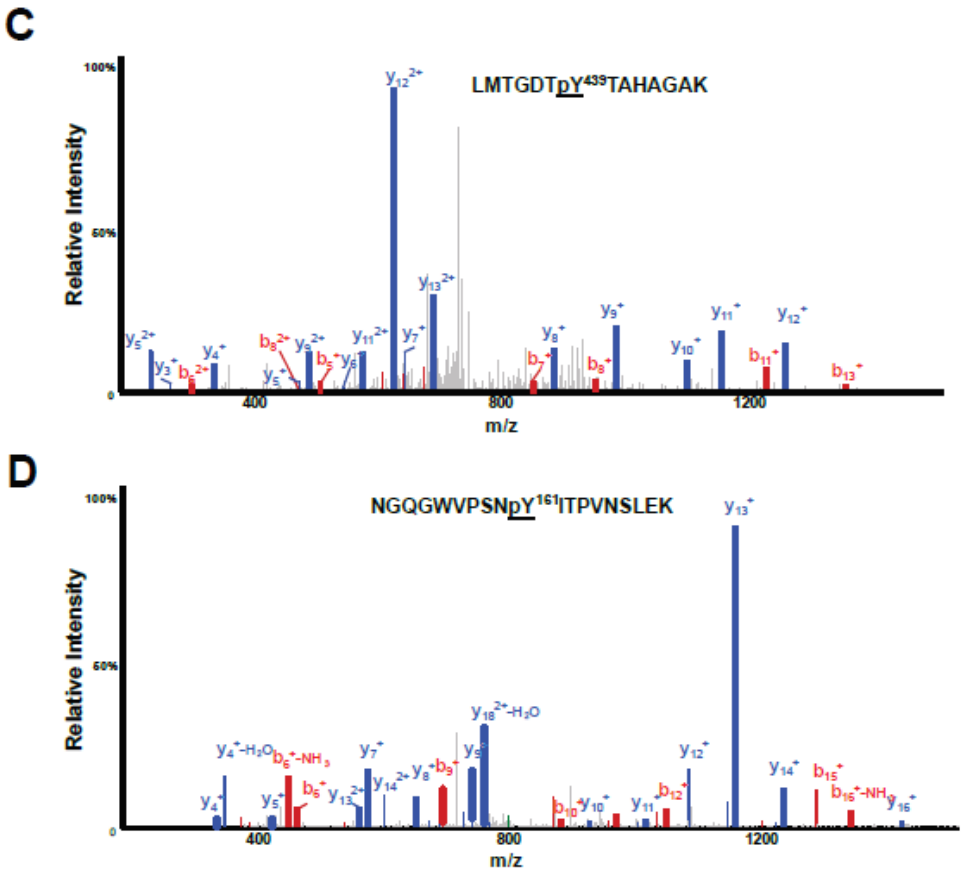


Figure 2.5: Phosphopeptide mapping with mass spectrometry identified novel phosphorylation sites (A) Phosphorylated Abl2 sample was prepared by incubating 1 μM of Abl2N (KI) with 10 nM of PDGFRβ and Mg2+/ATP. Reaction

samples were terminated with 4X LSB at 1, 2 and 4 hours and monitored for phosphorylation status. (top panel) 100 ng of the reaction sample was immunoblotted with antibodies to phospho-tyrosine (4G10). (bottom panel) 10 µg of reaction samples were separated by Phos-tag SDS-PAGE and visualized with Coomassie Blue stain. (B) Summary of tyrosine phosphorylation sites that are identified by mass spectrometry with Abl2N phosphorylated by PDGFRβ CD. (C) The MS/MS spectrum is shown for example of peptides containing Tyr439 with representative b and y fragment ions in red and blue respectively. (D) The MS/MS spectrum of peptides containing Tyr161 with representative b and y fragment ions in red and blue respectively.

In parallel with the mass spec study, we used smaller sub fragments of Abl2N as substrates to identify regions phosphorylated by PDGFRβ. We found that the isolated tandem SH3 SH2 domain fragment, a fragment of the SH2 domain containing the SH2-kinase linker, and the kinase domain were all phosphorylated by PDGFRβ, indicating that PDGFRβ can phosphorylate multiple sites as MS study suggests. To cross examined the MS study, we created a panel of Y to F substitution of phosphotyrosine identified by MS and also Y116 which was indicated by MS as a potential phosphorylation site but with lower confidence (Fig 2.6A). Mutation of tyrosine 272 (Y272F) on the SH2-kinase linker completely abrogated Abl2 SH2 domain phosphorylation by PDGFR, while the other triple mutant constructs did not reduce phosphorylation (Fig 2.6B). Mutation of three tyrosines in the SH3 domain (Y116F, Y139F and Y161) greatly reduces SH3-SH2 domain phosphorylation by PDGFR (Fig 2.6C). Mutations of four tyrosines in the kinase domain (Y299F, Y303F, Y310F and Y439F) also significantly reduced its phosphorylation (Fig 2.6D). Our mutagenesis kinase assay was consistent with MS findings. Interestingly, there are several novel phosphorylated tyrosine residues (Y116, Y161, Y272 and Y310) located at or near the SH3/SH2-kinase linker interface, which has an important regulatory role of keeping Abl family kinases in an auto-inhibited conformation (Fig 2.6E) (71). We hypothesize that PDGFRβ phosphorylation on these sites would disrupt the autoinhibitory binding interfaces between SH3 and SH2-kinase linker, resulting in Abl2 activation

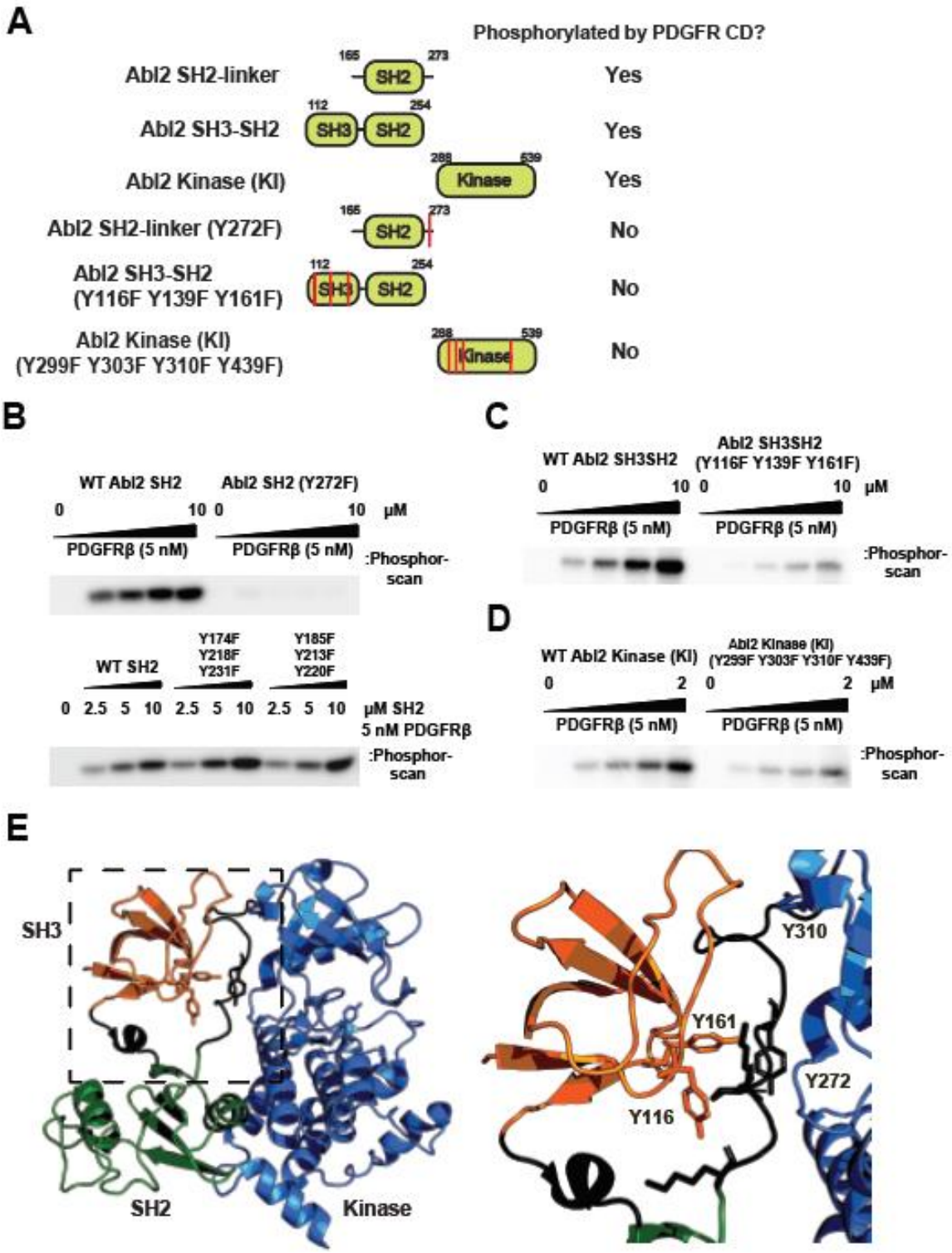


Figure 2.6: PDGFRβ phosphorylates Abl2 N-terminal half on several tyrosine residues at the SH3/SH2-kinase linker interface. (A) Domain

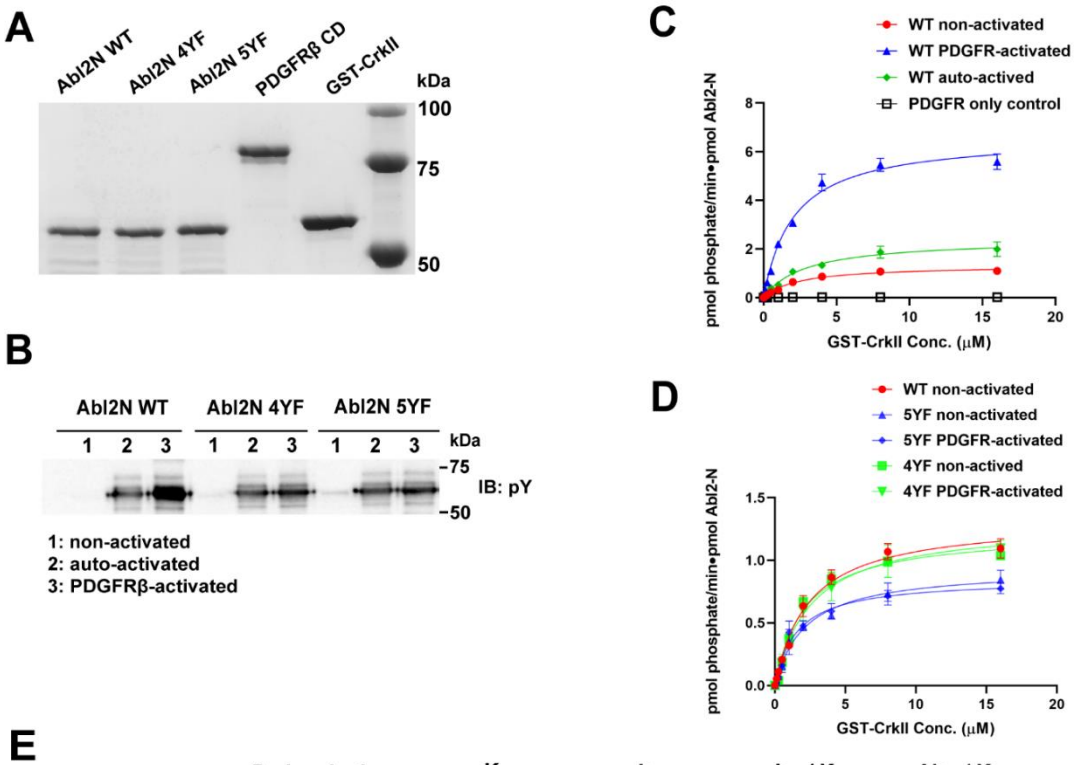
architecture of the smaller sub fragments of Abl2 N-terminal half and their mutant constructs (red lines indicate relative point mutation position). The table summarizes whether the construct is phosphorylated by PDGFRβ. (B) (top panel) Radioactive ATP Kinase assays of WT Abl2 SH2 domain and Abl2 SH2 (Y272F) mutant phosphorylated by PDGFRβ CD as kinase. (bottom panel) Radioactive ATP Kinase assays of WT Abl2 SH2 domain, and two SH2 triple mutant constructs. (C) Radioactive ATP Kinase assays of WT Abl2 SH3SH2 domain and Abl2 SH3SH2 (Y116F, Y139F and Y161F) mutant phosphorylated by PDGFRβ CD. (D) Radioactive ATP Kinase assays of WT Abl2 kinase (KI) domain and Abl2 Kinase (KI) (Y299F, Y303F, Y310F and Y439F) mutant phosphorylated by PDGFRβ CD. (E) (Left panel) Crystal structure of the autoinhibited state c-Abl1 N-terminus (PDB code 2FO0) (71). The position of SH3 domain (orange), SH2 domain (green), SH2-kinase linker (black), kinase domain (blue). (Right panel) Enlargement of the SH3/SH2-kinase linker interface. The SH2-kinase linker adopts a PPII helix that engage the SH3 domain. Abl2 Y116 and Y161 (Y89 and Y134 in Abl1) are located on the binding interface of SH3 domain that face the linker. Abl2 Y272 (Y245 in Abl1) is located on the linker and face the N-lobe of the kinase domain. Abl2 Y310 (Y283 in Abl1) is located on the kinase N-lobe and face the linker.

PDGFRβ phosphorylation activates Abl2 kinase activity.

Autophosphorylation of Y272 in Abl2 and phosphorylation of Y439 by Src family kinases promotes Abl2 kinase activity (46). We tested if PDGFRβ CD phosphorylation could activate the ability of Abl2 to phosphorylate its substrate CrkII in vitro, using purified proteins (Fig 2.7A). We first incubated 1 μM of Abl2N with 10 nM of PDGFRβ and Mg^{2+}/ATP in a 1-hour activation reaction during which we achieved significant tyrosine phosphorylation (Fig 2.7B). Control preparations include PDGFRβ only, Abl2N without PDGFRβ/ATP (non-activated), and Abl2N with ATP only (auto-activated) condition. Following these preincubations, we used 1 nM of Abl2N in kinase reactions to phosphorylate CrkII, and K_M and k_{cat} for the reaction were measured. The catalytic efficiency (k_{cat}/K_M) value for the non-activated Abl2N was determined to be 0.55 $\mu M^{-1}min^{-1}$ and autophosphorylated Abl2 had a k_{cat}/K_M = 0.83 $\mu M^{-1}min^{-1}$, while the PDGFR activated condition was 3.15 $\mu M^{-1}min^{-1}$. This result suggests that PDGFR phosphorylation on Abl2N promotes a 5.7-fold activation over baseline and a 3.8-fold activation over autophosphorylated Abl2N (Fig 2.7B, D)

To determine which tyrosine phosphorylation events contribute to Abl2 kinase activation, we next mutated five tyrosines that structural modeling suggested might be most relevant to Abl2 kinase activation (Y116F, Y161F, Y272F, Y310F and Y439F) to create the Abl2N 5YF construct. Incubation with the PDGFRβ CD did not result in Abl2N 5YF activation (Fig 2.7C, D). Src family kinase-mediated phosphorylation of Abl2 on Y439 phosphorylation within its activation loop promotes kinase activation (46). Hence, we also tested whether restoration of

Y439, in a 4YF mutant (Y116F, Y161F, Y272F and Y310F) impacts activation of Abl2N by PDGFRβ. Similar to the effects on the 5YF mutant, PDGFR phosphorylation did not result in Abl2 4YF activation (Fig 2.7C, D). These data suggest that PDGFR phosphorylation on one or more of the additional sites - Y116, Y161, Y272 and Y310 contributes to activation of Abl2N kinase activity.



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Kinase	Preincubation condition	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}μM^{-1}$)	$Δk_{cat}/K_M$ ($min^{-1}μM^{-1}$)
WT Abl2N	non-activated	2.41 ± 0.51	1.32 ± 0.08	0.55	1.00
WT Abl2N	auto-activated	2.90 ± 0.71	2.41 ± 0.22	0.83	1.60
WT Abl2N	PDGFR-activated	2.07 ± 0.32	6.63 ± 0.35	3.15	5.70
Abl2N (4YF)	non-activated	2.09 ± 0.41	1.23 ± 0.08	0.58	1.05
Abl2N (4YF)	PDGFR-activated	2.56 ± 0.59	1.29 ± 0.11	0.52	0.95
Abl2N (5YF)	non-activated	2.16 ± 0.52	0.94 ± 0.09	0.45	0.81
Abl2N (5YF)	PDGFR-activated	1.55 ± 0.37	0.85 ± 0.07	0.53	0.96

Figure 2.7. PDGFRβ phosphorylated Abl2 and modulate Abl2 kinase activation in vitro. (Figure caption on next page)

Figure 2.7. PDGFRβ phosphorylated Abl2 and modulate Abl2 kinase activation in vitro.

(A) Coomassie Blue-stained gel showing the purity of all recombinant purified protein used in this figure. (B) WT Abl2 N-terminus, 4YF (Y116F, Y161F, Y272F, Y310F) Abl2-N and 5YF (Y116F, Y161F, Y272F, Y310F, Y439F) Abl2-N were incubated with PDGFRβ in an activation reaction. Control conditions include Abl2-N constructs without PDGFRβ/ATP (non-activated), and Abl2-N constructs with ATP only (auto-activated). 100 ng of reaction product in all conditions were immunoblotted with antibody to phosphotyrosine. (C, D) Kinase activity was assayed by determining the kinetic parameter of GST-CrkII phosphorylation in [γ-32P] ATP kinase assays. Measurement collected along an increasing concentration (0-16 μM) of CrkII in each condition were fit to Michaelis-Menten isotherms using GraphPad. Error bar represent the S.E from n=3 concentration series for each condition. (E) K_M, k_{cat} and the catalytic efficiency (k_{cat}/K_M) values of Abl2-N mediated GST-CrkII phosphorylation were calculated from isotherms fit to perspective conditions shown in C and D.

PDGFRβ binds and phosphorylates Abl2 in cells.

We next investigated whether PDGFRβ binds and phosphorylates Abl2 in cells. PDGFRβ and Abl2-HA were co-expressed in HEK293 cells and stimulated with PDGF. Both WT and kinase inactive PDGFRβ and WT and R198K Abl2-HA expressed well at similar levels (Fig 2.8A). Only WT PDGFRβ underwent significant tyrosine phosphorylation following PDGF treatment (Fig 2.8A). We then immunoprecipitated Abl2 and measured Abl2 tyrosine phosphorylation levels. In cells expressing WT PDGFRβ, PDGF stimulation significantly increased Abl2 tyrosine phosphorylation levels by 2.2-fold, but similar increases in Abl2 tyrosine phosphorylation were not observed in PDGF stimulated cells expressing kinase inactive PDGFRβ (Fig 2.8B, C). Similarly, stimulation of the PDGFRβ did not increase tyrosine phosphorylation of the PDGFRβ – binding defective Abl2 R198K mutant. In fact, the basal tyrosine phosphorylation of the Abl2 R198K mutant was significant lower relative to WT Abl2. PDGFRβ also co-immunoprecipitated with Abl2 after PDGF stimulation, but complexes were not detected in transfections expressing either the PDGFR kinase inactive mutant or the R198K Abl2 mutant. These data suggest that PDGFRβ signaling promote Abl2 tyrosine phosphorylation and PDGFRβ/Abl2 interaction.

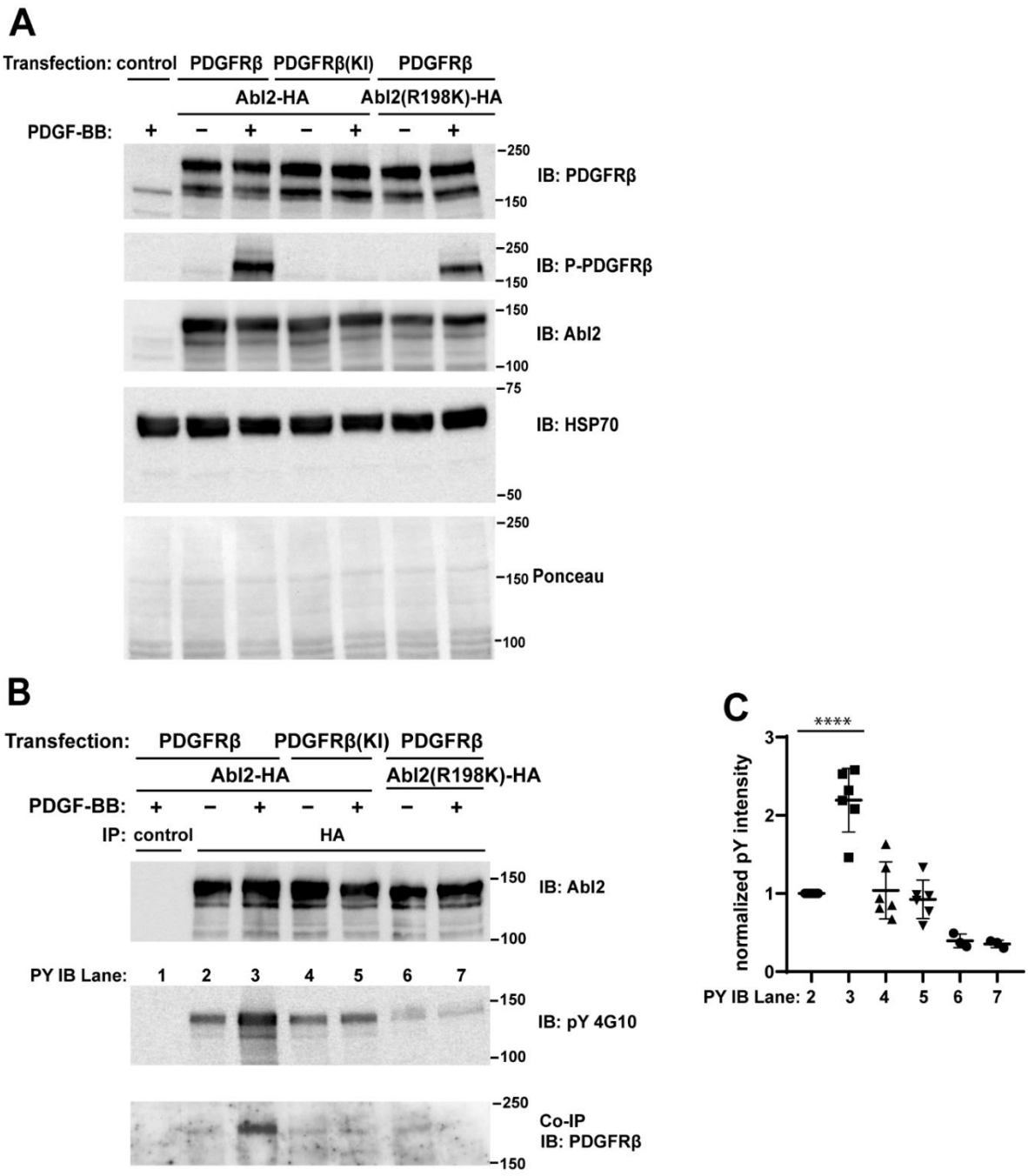

Figure 2.8 PDGFRβ form a complex with Abl2 and phosphorylates Abl2 in cells. (Figure caption on next page)

Figure 2.8 PDGFRβ form a complex with Abl2 and phosphorylates Abl2 in cells.

(A) HEK293 cells were either untransfected (control) or transfected with WT or kinase inactive (KI) PDGFRβ and WT or R198K Abl2-HA. HEK293 cells were serum starved overnight and treated with 100 ng/ml PDGF-BB for 10 minutes. 40 g of lysate were immunoblotted with antibodies to PDGFRβ, phosphotyrosine, Abl2 or HSP70. Bottom panel shows the Ponceau S-stained blots as loading controls. Molecular weight markers are indicated. (B) 500 mg of lysate in each condition was immunoprecipitated with anti-HA antibody. Control lane represent lysate pull down by protein-A/G beads without antibody. Immunoprecipitated product were blotted with antibodies to Abl2, phosphotyrosine and PDGFRβ. (C) Quantification of the normalized phosphotyrosine intensity shown in (B) from lane 2-7.

PDGFR activates Abl2 kinase activity in Fibroblasts

Finally, we addressed whether PDGFRβ activation leads to Abl2-mediated signaling events in cells. We used CRISPR in WT mouse 3T3 fibroblast cells to achieve 90% reduction in Abl1 levels and 88% reduction in Abl2 levels (Fig 2.9B). Stimulation of WT mouse fibroblasts with PDGF leads to an eight-fold increase in phosphorylation of the Abl1/Abl2 substrate CrkII (Fig 2.9A), but this was significantly abrogated in Abl1/Abl2 CRISPR double knock out (DKO) cells. However, we ascribe the small increase of CrkII phosphorylation after PDGF stimulation to this residual Abl1/Abl2 level in the DKO cells.

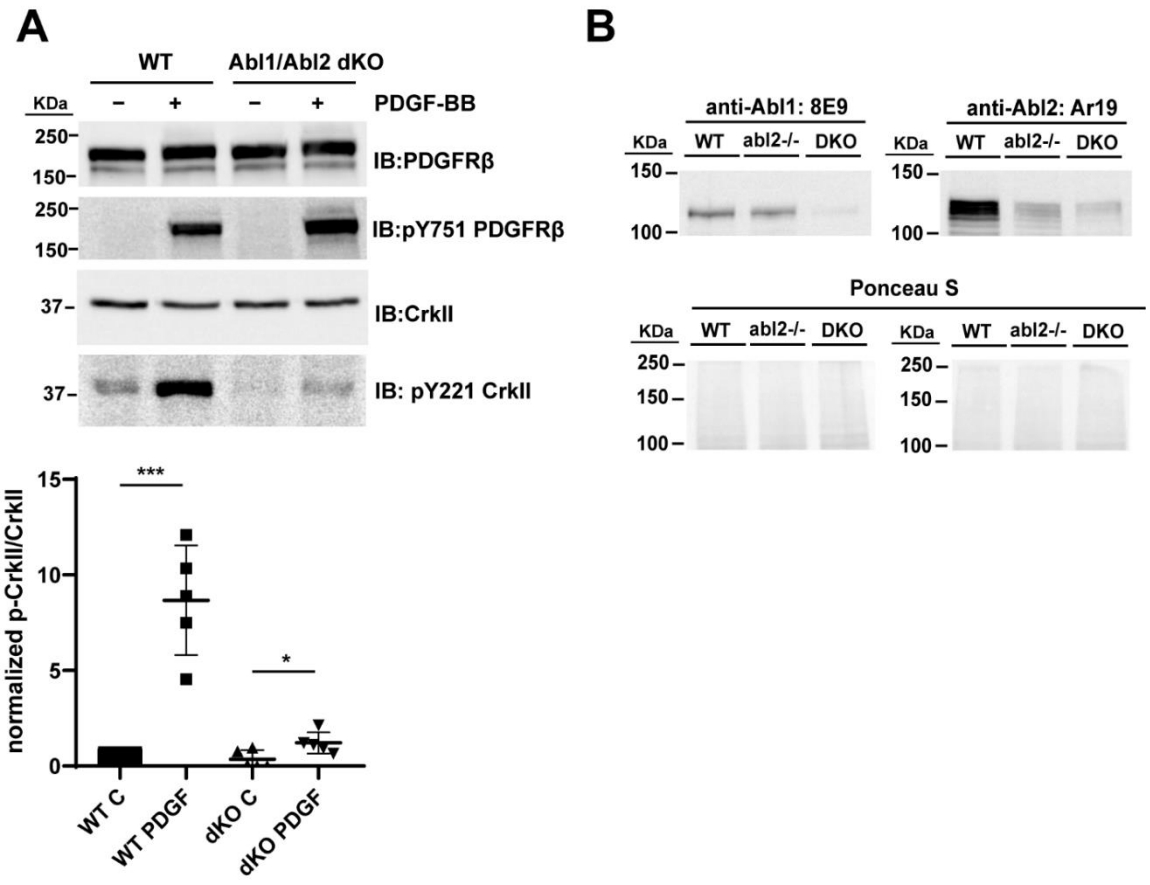

Figure 2.9 PDGFRβ signaling promotes Abl2 kinase activation in cells.

(A) (Top panel) WT and CRISPR Abl1/Abl2 dKO mouse 3T3 fibroblast cells were serum starved overnight and treated with 100 ng/ml PDGF-BB for 10 minutes. 40 µg of lysate were immunoblotted with antibodies to PDGFRβ, phosphotyrosine-751 in PDGFRβ, CrkII and phosphotyrosine-221 in CrkII. (Bottom panel) Quantification of normalized p-CrkII/CrkII intensity. (B) (Top Panel) WT, CRISPR Abl2 KO and CRISPR Abl1/Abl2 DKO mouse 3T3 fibroblast cells were immunoblotted with specific antibodies to Abl1 and Abl2. Bottom panel shows Ponceau S staining as loading control.

Discussion and future directions

We report here the molecular mechanisms by which PDGFRβ binds, phosphorylates, and activates the Abl2 kinase. We provide evidence that Abl2 binds to auto-phosphorylated PDGFRβ both in vitro and in cells via Abl2 SH2/ PDGFRβ phospho-Y771 interface. Abl2 recruitment results in PDGFRβ directly phosphorylating the Abl2 N-terminal half on multiple sites. Using both kinase assays with Tyr to Phe substitution Abl2 constructs and phosphopeptide mapping with mass spectrometry, we identified up to 8 phosphotyrosine sites on Abl2. We demonstrated that PDGFRβ phosphorylation on Abl2 results in Abl2 kinase activation both in vitro and in cells. These findings provide a molecular mechanism to understand how receptor tyrosine kinases activate Abl family kinase through different phosphorylation events.

PDGFRβ may serve as a scaffold to coordinate Abl kinase activation with other signaling outputs.

PDGF stimulation induces homodimerization of PDGFRβ as well as heterodimerization of PDGFRα and PDGFRβ, resulting in receptor autophosphorylation at multiple sites. These autophosphorylated Tyr residues serve as docking sites to recruit and activate multiple SH2 domain- containing singling proteins to elicit specific cellular responses (37,38). Some of these effectors have intrinsic enzymatic activities including Src family kinases (SFK),

phospholipase C- γ (PLC-γ), Ras-GTPase activating protein (Ras-GAP) and Src homology phosphatase 2 (SHP2) (37-41). Among them, both SFKs and PLC-γ had previously been shown to active Abl family kinases through different mechanisms (1-3,25,26). Interestingly, SFKs bind sites on PDGFRβ (pY579/Y581) that are distinct from the pY771 that recruits Abl2. Thus, the PDGFRβ may serve as a scaffold to bring these proteins in proximity to promote Abl2 activation. The dimerized form of PDGFRβ may also facilitate Abl2 Y272 autophosphorylation in trans, which also promotes kinase activation (25,26). We anticipate that other cell receptors that activate Abl family kinases including EGFR and integrin (4,9,21,42,43) may similarly use phospho-Y residues to recruit the Abl kinases and co-regulators, thereby acting as platform for Abl family kinase activation.

The identification of phospho-Y771 on PDGFRβ as an Abl2 binding site also has implications for additional possible modes of Abl2 kinase regulation. For example, the SHP2 tyrosine phosphatase specifically dephosphorylates Y771 in PDGFRβ, which may restrict Abl recruitment to PDGFRβ (44). In addition, Ras-GAP also binds to pY771 (40) and it may compete with Abl2 and limit PDGFRβ-mediated Abl2 kinase activation by competing with Abl2 recruitment to the receptor.

Disruption of the SH3/SH2-kinase linker interaction through phosphorylation may be a common mechanism in Abl kinase activation

In the inactive state, the Abl SH3 domain binds to the proline rich linker between the SH2 and kinase domains, which adopt a polyproline type II (PPII) helical conformation (28,45,46). Mutations of the SH3 domain and the linker prolines perturbs this this intramolecular interaction thereby activating Abl kinase activity (30,47,48). We found that PDGFRβ phosphorylates Abl2 on four interesting tyrosines (Y116, Y161, Y272 and Y310) that are all located at or near the SH3/SH2-kinase linker interface, which is critical to keep Abl family kinases in an auto-inhibited conformation (Fig 2.6D) (28). Engagement of the Abl2 SH2 domain with PDGFRβ is proposed to disengage this autoinhibited conformation. Subsequent phosphorylation on one or more of these sites would prevent reengagement of SH3 domain with the SH2-kinase linker and shift Abl2 into a non-inhibited “open” activated conformation. Consistent with this, previous studies demonstrated that phosphorylation of Abl2 Y116, Y161 and Y272 (Y89, Y134 and Y245 in Abl1) or mutation of key P residues in the SH2 kinase linker prevent engagement of Abl SH3 domain with the SH2-kinase linker and are associated with enhanced Abl kinase activity (26,48-50). Abl2 SH3 domain phosphorylation may also result in such extended conformation, which release the SH2-kinase linker. This “opening” of the kinase likely also promotes the autophosphorylation of the Y272 (Y245 in Abl1), an important step in Abl kinase activation (25,26).

Future experiments are needed to determine the role of other phosphorylation sites on Abl2 by PDGFRβ. Interestingly, Y299 and Y303 are both located on a highly conserved glycine rich P-loop in the kinase domain that contacts the phosphate group of ATP. Their mutations had been implicated in Bcr-Abl resistant to STI-571 (84,106,107). Phosphorylation of these sites may potentially stabilize the active kinase conformation. Other phosphorylation sites may serve as binding interface for SH2 containing proteins that may have regulatory role for Abl kinases.

Abl family kinase activation as a multi-step process

Phosphorylation of Abl2 at Y439 (Y412 on Abl1) in the kinase activation loop is a critical step for full kinase activation of Abl family kinases (26,48). However, how this process is regulated is not fully understood. We show that PDGFRβ CD phosphorylation on the Abl2 N-terminal half (Abl2N) promotes a 5.7-fold activation over non-activated Abl2N in vitro. Mutation of Y116, Y161, Y272, Y310 and Y439 (5YF) abrogated PDGFRβ-mediated activation of Abl2 kinase activity. Surprisingly, an Abl2N mutant in which Y439 was restored (e.g. the 4YF mutant - Y116F, Y161F, Y272F and Y310F) could not undergo activation by PDGFRβ. These data suggest either that Y439 is not efficiently phosphorylated when Abl kinase remains in a “closed” inactive conformation or that Y439 phosphorylation itself does not activate the kinase when the SH3 and SH2 domain remain in the

auto-inhibited conformation. Our model may provide some insight that activation loop phosphorylation and kinase activation may be regulated by N-terminal domain conformation.

Future experiments are needed to quantify the extend of activation loop phosphorylation under different Abl kinases conformations. Phosphorylation on the SH3 and SH2-kinase linker may initiate the transformation of Abl kinases from an auto-inhibited state to an extend state. This process may be required for phosphorylation on the activation loop to function as it stabilized the active kinase.

Our work adds to a growing body of data indicating that Abl kinases are not simply switched between a closed auto-inhibited state and an open-active state through a one-step process (9,25,26,29). Instead, Abl kinases appear to be regulated by different types and degrees of intermolecular/ intramolecular interactions and post-translational modifications across a spectrum of activity levels (25-29). Our findings provide a mechanism to understand how Abl kinases are precisely regulated through multi-steps phosphorylation events by receptor tyrosine kinases.

CHAPTER 3- PDGFR signaling regulate integrin activation through Abl2

Abstract

Integrin adhesion receptors undergo rapid and reversible conformational changes to regulate their affinity for extracellular ligands (91,108). This inside-out signaling process, known as integrin activation, is achieved by binding of the intracellular proteins talin and kindlin to the cytoplasmic tail of the integrin β subunits (91,108). Regulated activation of integrin is critical for cell adhesion, motility and tissue homeostasis (91,108). One understudied area of importance is growth factor modulation of integrin signaling. A variety of growth factors including PDGF, are known to modulate integrin signaling and activation (109-114). However, the precise molecular mechanisms and signaling pathways mediating growth factor regulation of integrins are as of yet not elucidated. Our lab has demonstrated that β1-containing integrins signal through Abl2 nonreceptor tyrosine kinase (44,88,115). We recently showed that Abl2 is activated through direct interaction with integrin β1 cytoplasmic tail. Interestingly, the Abl2 binding interfaces on integrin β1 overlap with those of talin, a key integrin activator (44). Hence, it is possible Abl2 may regulate talin-integrin binding, and potentially act as an integrin inactivator. PDGFR can also directly interact with Abl2 and activate its kinase activity, and my preliminary data suggest that PDGFR may influence integrin activity through Abl2.

Introduction

Integrins are a large family of transmembrane adhesion receptors that provide a dynamic and bi-directional structural and signaling connection between the extracellular matrix and intracellular cytoskeleton (91,108). Integrin receptors are heterodimers comprised of α and β subunits, and they can shift between an inactive bent conformation and an active extended conformation (91,108). The binding of talin to the β integrin cytoplasmic tails induces conformational changes of the integrin extracellular domain, which results in an increased affinity for extracellular ligand binding (108,116-121). This process is known as integrin activation, and it provides a rapid and reversible mechanism that regulates integrin activity, which is crucial for cell adhesion, migration and differentiation (108,116-121). Structural studies show that the talin F3 domain interacts with the integrin β1 membrane proximal region through several key residues including two phenylalanine and one tryptophan residue, as well as the membrane proximal NPXY motif (118-121). Furthermore, talin binding to integrin β1 disrupts interactions between the cytoplasmic membrane proximal regions of integrin α and β, separating them and allowing them to interact with other intracellular binding partners. This inside-out process also induces integrin receptors to adopt an extended conformation leading to a higher affinity for extracellular matrix ligands like fibronectin (108,116-121).

Competitive inhibition of talin binding to the integrin cytoplasmic domain provides a common mechanism to regulate integrin activity (117,122-128). For example, the immunoglobulin-like domain of filamin A directly interacts with the integrin β

tail membrane proximal NPXY motif and competes away talin, which results in inhibition of talin-dependent integrin activation (125). Tyrosine phosphorylation of the integrin β tail is also a conserved mechanism for regulating integrin activation. Studies show that the binding affinity between talin and integrin β3, β1A, and β7 is greatly decreased by tyrosine phosphorylation (102,103). Our lab recently showed that Abl2 kinase domain directly binds the lysine-rich region on integrin β1 tail, and phosphorylates integrin β1 on the membrane proximal NPXY motif (44). Interestingly, the binding interfaces on integrin β1 overlap with those of talin (44). Abl2-mediated tyrosine phosphorylation on integrin β1 provides additional binding site for Abl2 through its SH2 domain. Hence, Abl2 may inhibit talin-dependent integrin activation through either direct competition in binding or phosphorylation. There are extensive examples of cross-talk between growth factor receptor and integrin (109-111,114,129,130). Emerging data from our lab and others have shown that PDGF stimulation may have a role in regulating integrin activation through Abl2. However, the role of Abl2 and the molecular mechanism underlying the crosstalk between PDGFR and integrin remains unknown. Here, I report initial studies that suggest Abl2 relays PDGFR signaling to regulate integrin activation through inhibiting talin-integrin β1 interaction as this model shows (Fig 3.1).

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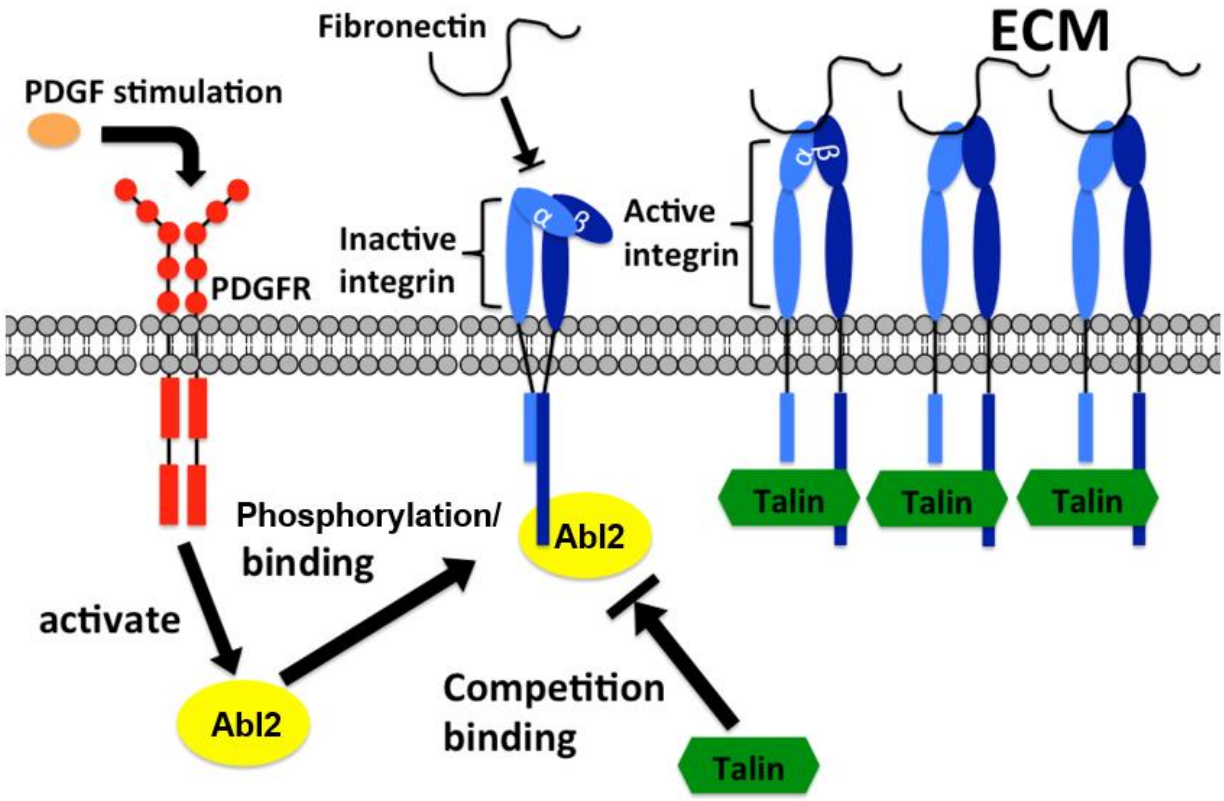

Figure 3.1 Model of the molecular mechanism by which PDGFR signaling regulate integrin activation through Abl2

PDGFR signaling activates Abl2 kinase, promotes Abl2-integrin β1interaction and Abl2 mediated phosphorylation on integrin β1. This process competes away talin for integrin binding and results in inhibition of talin-dependent integrin activation.

Results

Abl2 competes with talin for integrin β1 interaction in cells upon PDGF stimulation

To test whether Abl2 competes with talin for integrin β1 binding in cell, we used Fluorescence Resonance Energy Transfer (FRET) by Fluorescence Lifetime Imaging Microscopy (FLIM) to measure their interactions in collaboration with Dr. Maddy Parsons. We found that FRET between Abl2 and β1 is robustly elevated upon PDGF stimulation. Furthermore, this interaction is attenuated in cells expressing β1 4KA (four lysine to alanine in the lysine rich motif, which significantly reduces Abl2 binding) mutant (Fig 3.2A, B). Using similar methods, we investigated the effect of PDGF stimulation on Abl2/talin competition in cells. *β1-/-* mouse embryonic fibroblasts expressing β1-GFP and either talin-mRFP or kindlin-mRFP in the presence or absence of Abl2-HA were stimulated with PDGF (Fig 3.2C, D). FRET between β1 and either talin or kindlin were monitored using FLIM. Kindlin is another integrin activator, but unlike talin, it engages the β tails membrane distal NPxY/F motif whereas talin binds to the membrane proximal of these motifs (131). We found that Abl2 overexpression led to a strong decrease in FRET between β1 and talin, but not β1 and kindlin in response to PDGF stimulation (Fig 3.2 C, D). Our data indicates that Abl2 specifically competes with talin but not kindlin for β1 binding in cells. I also designed a small hairpin RNA targeting Abl2 to knock down endogenous Abl2 expression levels (Fig 3.2 E). Our results show that PDGF stimulation led to a significant reduction in FRET between β1 and talin in control cells, but not in Abl2 knock down cells (Figs

3.2E). Together, these data strongly suggest that Abl2 specifically competes with talin for integrin β1 interaction in cells.

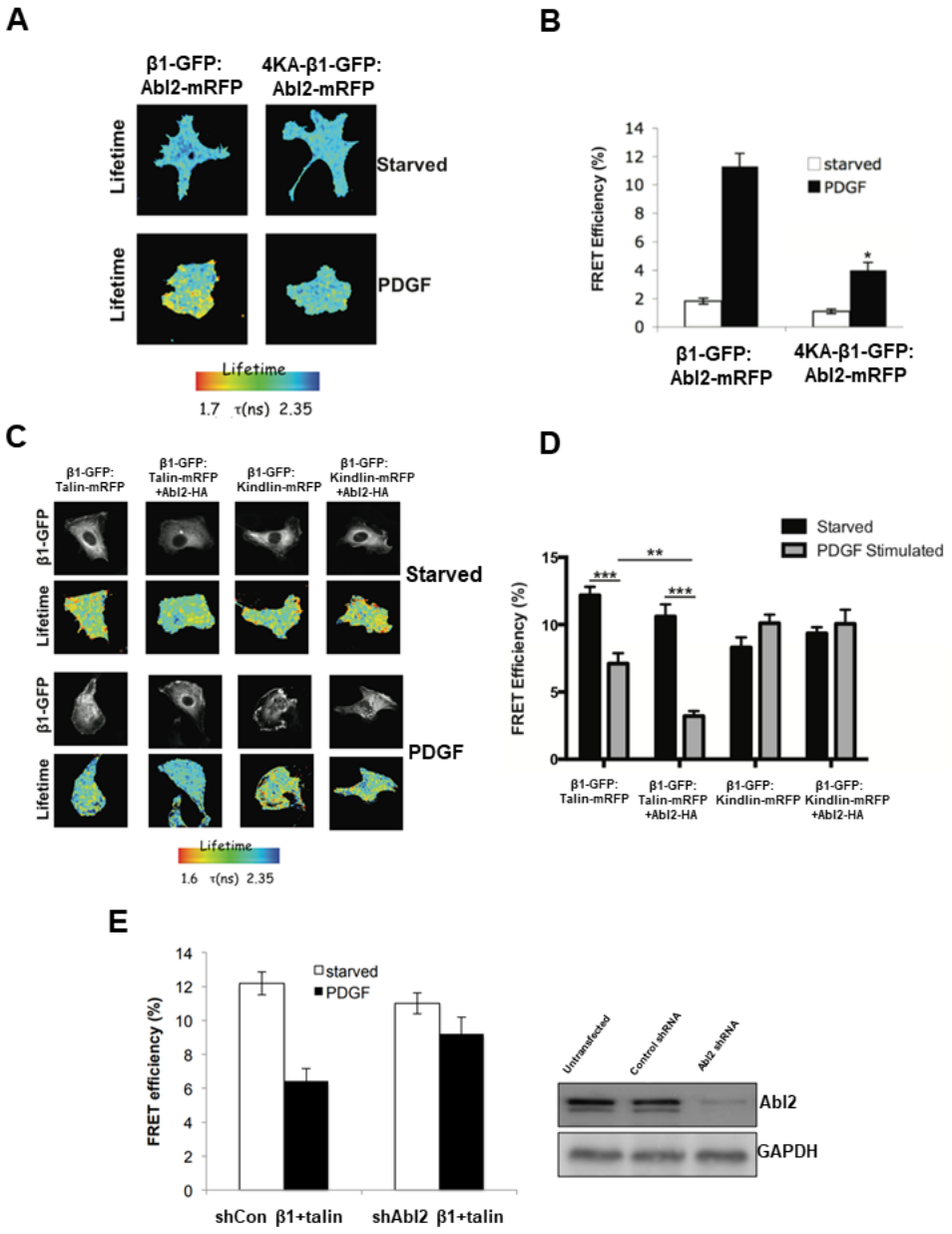

Figure 3.2 Abl2 competes with talin for integrin β1 interaction in fibroblast

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Figure 3.2 Abl2 competes with talin for integrin β1 interaction in fibroblast

(A) *β1-/-* mouse embryonic fibroblasts (MEF) expressing Abl2-mCherry and either β1-GFP or 4KA-β1-GFP were serum starved and then stimulated with PDGF to monitor the ability of Abl2 to interact with β1 upon PDGF simulation. PDGF enhances Abl2's ability to interact with β1, but not the Abl2 kinase interaction deficient β1 mutant, 4KA. (B) Quantification of FLIM-FRET from (A). (C) *β1-/-* MEFs expressing β1-GFP and either talin-mRFP or kindlin-mRFP in the presence or absence of Abl2-HA expression were either serum starved or stimulated with PDGF before monitoring FRET by FLIM between β1 and either talin or kindlin. Abl2 overexpression attenuates talin, but not kindlin, interactions with β1 upon PDGF stimulation. (D) Quantification of FLIM-FRET from (C). (E) (left panel) Quantification of FLIM-FRET of *β1-/-* MEFs either transfected with control shRNA or Abl2 shRNA expressing β1-GFP and talin-mRFP were serum starved and stimulated with PDGF before monitoring FRET by FLIM between β1 and talin. Western blot of MEFs untreated or transfected with control shRNA or Abl2 shRNA probed for Abl2 (top) or GAPDH (bottom).

PDGF stimulation promotes integrin β1Y783 phosphorylation by Abl2

Previous work from my former colleague Dr. Adam Simpson established that Abl2 phosphorylates integrin β1 cytoplasmic domain on Y783. PDGF stimulation not only activates Abl2 kinase, but also promotes Abl2-integrin β1 interaction in cells. I hypothesized that PDGF simulation promotes integrin phosphorylation by Abl2. To address this question, WT mouse 3T3 fibroblast cells were starved overnight and treated with Mn^{2+} and PDGF, and lysates were blotted with a phospho-specific antibody to pY783 in integrin β1. Mn^{2+} has been widely used to simulate integrin activation in the absence of a bound ligand, potentially making β1 cytoplasmic domain more accessible for cellular binding partners (132). My results show that treatment of Mn^{2+} to activate integrins increases integrin β1 Y783 phosphorylation, which is consistent with previous findings (44). PDGF stimulation after Mn^{2+} treatment leads to higher β1 Y783 phosphorylation levels compared to Mn^{2+} treatment alone while integrin β1 expression levels remained the same. (Fig 3.3A). To address whether Y783 phosphorylation is dependent on Abl2 kinase activity, I transiently transfected HEK293 cells with YFP control, Abl2-YFP and Abl2 (kinase inactive)-YFP, and measured integrin β1 Y783 phosphorylation following Mn^{2+} and PDGF treatment. My results show that Mn^{2+} and PDGF treatment led to a slight increase in integrin β1 phosphorylation in YFP transfection control cells due to low endogenous Abl2 levels, whereas WT Abl2 expression greatly increased β1 phosphorylation levels (Fig. 3.3B). However, kinase inactive Abl2 expression does not increase Y783 phosphorylation level compared to YFP transfection control cells (Fig. 3.3B).

These results suggest that PDGF stimulation promotes Abl2 mediated integrin β1 Y783 phosphorylation.

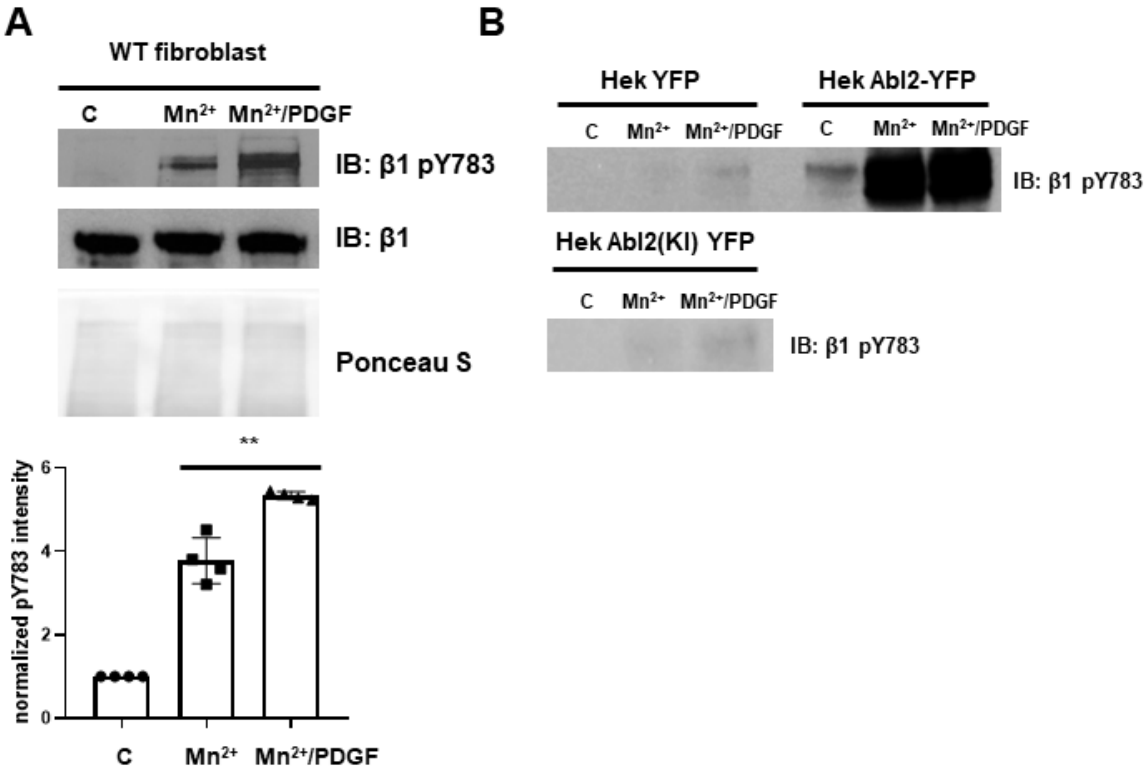


Figure 3.3 PDGF stimulation promotes integrin β1 phosphorylation by Abl2

(A) WT mouse 3T3 fibroblast cells were serum starved overnight and treated with 2mM $MnCl_2$ for one hour before stimulated with 100 ng/ml PDGF-BB for 10 minutes. 40 µg of lysate were immunoblotted with antibodies to integrin β1 pY783 and integrin β1. Ponceau S staining was used as loading control. Bottom panel shows the quantification of normalized phosphotyrosine 783 intensity in different conditions (n=4 for each condition, ** = $p < 0.01$). (2) HEK93 cells expressing YFP control, Abl2-YFP or Abl2 (Kinase inactive)-YFP were serum

starved overnight and treated with 2mM $MnCl_2$ for one hour before stimulated with 100 ng/ml PDGF-BB for 10 minutes. 40 µg of lysate were immunoblotted with antibodies to integrin β1 pY783.

Abl2 and talin compete for integrin β1 binding in vitro

Previous work from our lab established that Abl2 directly engages integrin β1 cytoplasmic tail via two distinct interaction interfaces (44). The Abl2 kinase domain directly interacts with a lysine-rich membrane-proximal segment in the integrin β1 cytoplasmic tail, and it phosphorylates Y783 in the membrane proximal NPxY motif of β1 (44). Abl2 SH2 domain can then engage the phosphorylated NPxY motif in the integrin β1 tail (44). Given that these binding interfaces overlap with those of talin, I hypothesized that Abl2 competes with talin for binding to the integrin β1 tail (Fig 3.4A). To directly test this hypothesis, I performed in vitro single competitor competition binding assay with talin F3 domain linked beads incubated with an increasing concentration gradient of GST-β1 and either BSA or Abl2 N-terminal half (Abl2N) as competitor (Fig 3.4B). GST-β1 tail retained on the talin F3 beads significantly reduced when Abl2N was used as competitor compared to BSA (Fig 3.4B). This result indicates that Abl2 and talin compete for integrin β1 binding. Furthermore, I performed a similar experiment with constant concentration of GST-β1 tail in the supernatant and an

increasing concentration gradient of Abl2N as competitor (Fig 3.4C). The increasing concentration gradient of Abl2N resulted in a dose dependent decrease in β1 tail retaining on talin-linked beads, further validating that there is a direct competition between Abl2 and talin for integrin β1 binding.

Note that the recombinant β1 tail is not phosphorylated in this experiment and thus, talin F3 likely sterically blocks binding of Abl2 to the β1 lysine rich interface. Abl2-mediated phosphorylation on the β1 NPxY motif would also influence the competition between Abl2 and talin for binding to the β1 tail, both by potentially increasing Abl2 affinity for the β1 tail, and by decreasing affinity of the β1 tail for talin (102,103). Therefore, β1 phosphorylation may shift the Abl2/talin competition equilibrium towards Abl2. To directly test whether integrin β1 Y783 phosphorylation affects Abl2 and talin interaction, I performed fluorescence anisotropy (FA) binding assay with fluorescein isothiocyanate (FITC) tagged Y783 phosphorylated and non-phosphorylated synthesized integrin β1 peptides. The integrin β1 peptides (758D-787V) contain binding interfaces for both Abl2 and talin (Fig 3.4D). A constant concentration of FITC labeled β1 peptides were titrated with an increasing concentration gradient of GST-Abl2 SH2 domain, talin F3 domain, and GST as a control (Fig 3.4D). Protein-protein interaction measured by change of FA between pY783 β1 peptide and Abl2 SH2 domain reached saturation, while change of FA between GST/pY783 β1 and Abl2 SH2/non-phosphorylated β1 remained at low background level, as expected. To ensure bona fide changes of FA resulting from protein-protein interactions, a control competition experiment with untagged-pY783 β1 was performed (Fig

3.4E). In this experiment, 5 nM of FITC-pY783 β1 was incubated with 5μM of GST-Abl2SH2 and an increasing concentration gradient of unlabeled pY783 β1 peptide. The increasing concentration of unlabeled pY783 β1 peptide resulted in a dose dependent decrease in FA, further validating that the change of FA results from interaction between FITC-pY783 β1 and Abl2 SH2 domain. The changes of FA between talin F3 domain and both pY783 β1 binding and unphosphorylated β1 binding increases as talin F3 concentration increases, but did not reach saturation (Fig 3.4D). There is no significant difference in binding between pY783 β1 and unphosphorylated β1 binding to talin F3 domain.

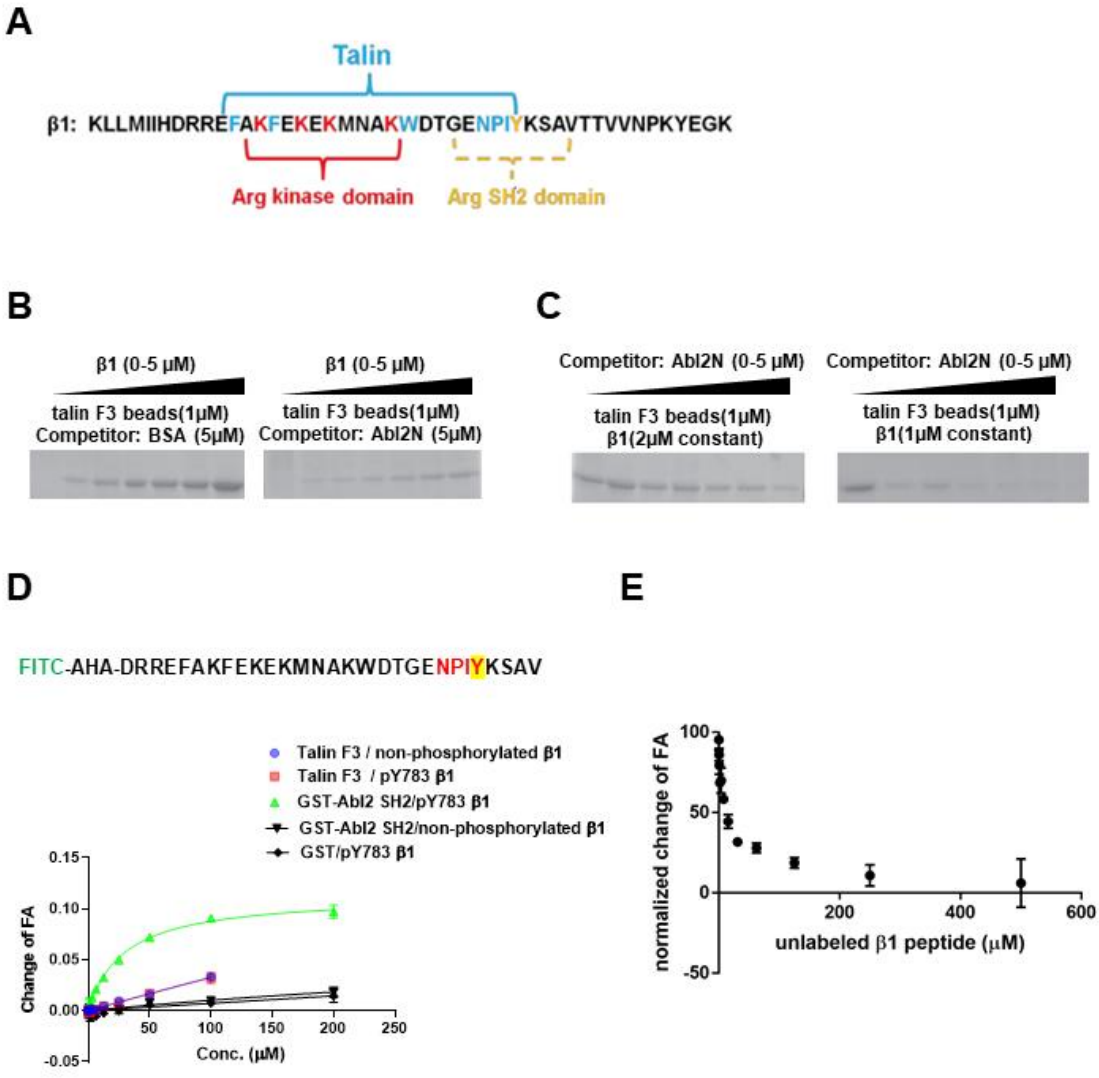


Figure 3.4 Abl2 and talin compete for integrin β1 binding

(A) Talin-β1 binding interface (blue) overlaps with Abl2 kinase and SH2 domain binding interfaces (red and yellow). (B) 1μM of talin F3 domain linked beads were incubated with 0-5 μM of GST-β1 integrin tail and 5 μM of either BSA or Abl2 N-terminal half as

competitor. (C) 1μM of talin F3 domain linked beads were incubated with 1 or 2 μM of GST-β1 integrin tail and a concentration gradient (0-5 μM) of Abl2N as competitor. (D) (top panel) sequence of the FITC-tagged β1 peptide with the

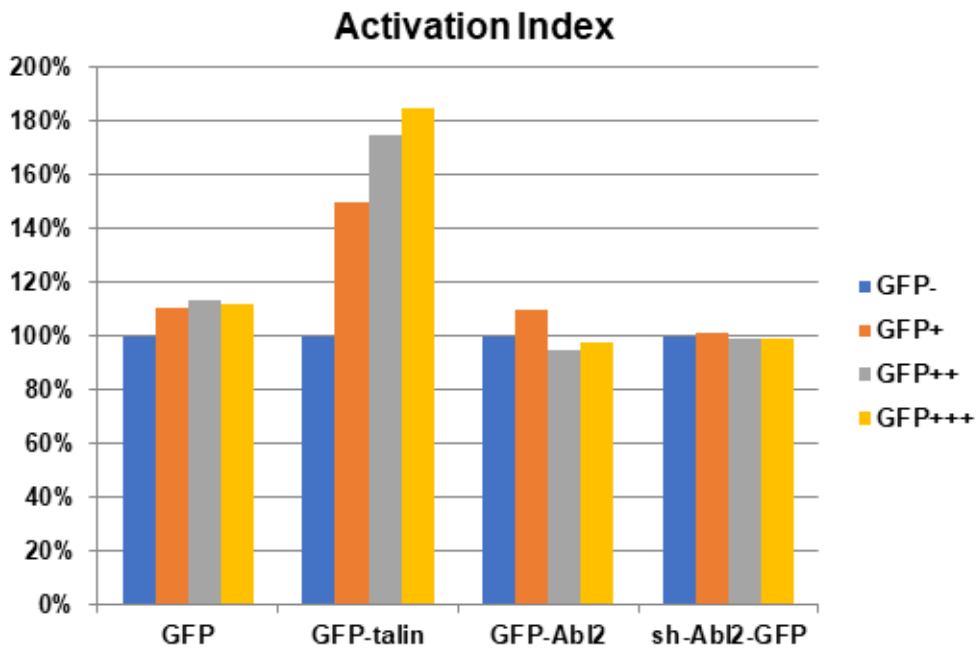
membrane proximal NPXY motif highlighted in red. One of the peptides is phosphorylated on the NPXY tyrosine (Y783) (bottom panel) 5nM of FITC tagged pY783 or non-phosphorylated β1 peptide were incubated with an increasing concentration gradient of talinF3 domain, GST-Abl2SH2 domain and GST as a control. Change of FA is measured and plotted against the concentration of binding partners. (E) 5nM of FITC-pY783 β1 peptide was incubated with 5 μM of Abl2 SH2 domain and an increasing concentration gradient of unlabeled pY783 β1. Change of FA is measured and plotted against unlabeled pY783 β1 concentration.

Investigate the effects of PDGF-driven Abl2 binding on integrin activity

Our FRET-FLIM data suggest that PDGF stimulation strongly reduces talin-β1 interaction through Abl2 signaling (Fig 3.2). Since talin is critical for integrin β1 activation, I hypothesized that PDGF stimulation may reduce the relative levels of integrin α5β1 activation. In collaboration with Dr. Daniel Iwamoto in Dr. David Calderwood's lab, we assessed the activation state of endogenous α5β1 by measuring the binding of a fibronectin fragment (FN9-11) to activated integrin in a Fluorescence Activated Cell Sorting (FACS) based assay as previously described (116,133). Briefly, after transfection or PDGF treatment, cells were suspended and incubated with FN9-11, a soluble fibronectin fragment that binds

to activated integrin α5β1. The activity of integrin in each experiment was determined by activation index with the following equation: $\mathrm{AI} = (\mathrm{F} - \mathrm{F0})/\ (\mathrm{F\ integrin})$; where F is the mean fluorescence intensity (MFI) of FN 9-11 binding, and F0 is the MFI in presence of EDTA to prevent ligand binding. F integrin is the normalized MFI of integrin antibody PB1 which determines total surface integrin β1. We transfected Abl2 and talin (1-433) in CHO cells and investigated how Abl2 and talin expression affects integrin activation. As expected, talin (1-433) expression induced integrin activation, and high integrin activity is correlated with high talin expression levels (Fig 3.5A). However, Abl2 expression does not affect integrin activation. We also transfected a small hairpin RNA targeting Abl2 to knock down endogenous Abl2 expression levels. Abl2 knock down also did not have any significant effect on integrin activation (Fig 3.5A). Next, we tried to investigate whether PDGF stimulation affects integrin activation through Abl2 signaling. WT or Abl2 knock out (KO) fibroblasts were serum starved overnight and stimulated with different doses of PDGF (10ng/ml, 30ng/ml and 100ng/ml). Surprisingly, PDGF stimulation has no significant effect on either WT or Abl2 KO fibroblast. Future experiments will be required to investigate the biological importance of Abl2 mediated inhibition of talin-integrin binding and identify if there are any additional players involved in this pathway.

A



B

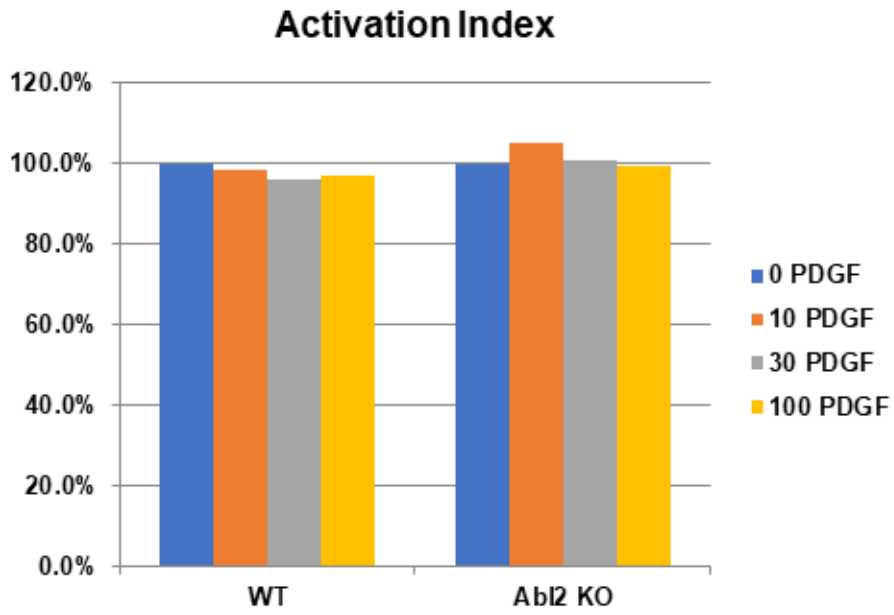


Figure 3.5 Abl2 expression or PDGF stimulation have no significant effect on β1 **integrin activation** (Figure caption on next page)

Figure 5.5 Abl2 expression or PDGF stimulation have no significant effect on β1 integrin activation

(A) CHO cells were transfected with GFP-talin (1-433), GFP-Abl2, GFP-shAbl2 and GFP as transfection control. 24hour post-transfection, cells were detached, washed and GFP expression and binding of FN-9-11 to live cells in the presence or absence of EDTA were analyzed by FACS. α5β1 integrin expression was assessed in parallel by staining with PB1. Integrin activation index was determined with the following equation: AI=(F-F0)/ (F integrin); where F is the mean fluorescence intensity (MFI) of FN 9-11 binding, and F0 is the MFI in presence of EDTA to prevent ligand binding. F integrin is the normalized MFI of integrin antibody PB1 which determines total surface integrin β1 (B) WT and Abl2 KO mouse fibroblasts were serum starved overnight and stimulated with different dose of PDGF for 10 minutes Cells were detached, washed and the activation index of endogenous α5β1 integrin was assessed as described in (A).

Discussion and future direction.

Here I report a putative molecular mechanism by which PDGFR signaling regulates integrin activation through Abl2-mediated inhibition of talin-integrin β1 interaction. This could be achieved through either talin-Abl2 direct binding competition or integrin β1 Y783 phosphorylation by Abl2. Direct binding competition and phosphorylation events are common mechanisms to regulate integrin activity by many integrin inhibitors (102,103,122-124). My initial findings suggest thsat PDGF simulation promote Abl2-integrin β1 interaction and β1 Y783 phosphorylation by Abl2. PDGF stimulation also significantly reduces integrin β1/talin interaction, and the reduction is directly related to Abl2 expression level. Inhibition of talin-integrin β1 interaction by other integrin inhibitors regulates integrin activation, which is crucial for proper cell migration and adhesion. Abl2 acting downstream of PDGFR to regulate integrin activation may provide a mechanism to allow PDGFR to crosstalk with integrin to regulate cancer metastasis, angiogenesis and embryonic development (110,134-136). My initial findings demonstrate that there are more players and factors involved in the downstream PDGFR stimulation-mediated Abl2 regulation, leaving many doors open for future studies to further dissect this complex signaling pathway.

What is the role of Abl2 mediated integrin phosphorylation on talin-integrin interaction?

We found that Abl2 overexpression led to a strong decrease in interaction between β1 and talin upon PDGF stimulation. However, whether the disruption of talin-integrin β1 binding is mediated through Abl2 binding and/or phosphorylation is not known. Using Abl kinases specific inhibitors like GNF-2 or GNF-5 in FRET-FLIM experiments to investigate whether Abl2, with its kinase activity inhibited, can disrupt talin-integrin β1 interaction remains unexplored. Using Abl2 KO fibroblasts rescued with kinase inactive Abl2 (K317M) will also be useful to evaluate the importance of Abl kinases activity in disrupting talin-integrin interaction. Previous studies show that talin F3 domain engages the integrin β1 tail through several key residues, but the membrane proximal NPXY motif is particularly important. Phosphorylation of NPXY tyrosine inhibits talin binding (102,103). A single tyrosine to alanine mutation in the β1 NPXY motif prevents talin binding and affects cell adhesion, spreading, and migration. (103,121,133,137,138) Mice bearing this mutation result in β1 null-like peri-implantation lethality (138). Therefore, it is expected that Abl2 mediated phosphorylation of β1 membrane proximal NPXY Tyr783 would inhibit talin binding.

I used chemically synthesized nonphosphorylated and pY783 integrin β1 peptide in fluorescence anisotropy (FA) experiments to test the effect of Y783 phosphorylation in talin binding. Based on my FA measurements, the dissociation constants (K_D) of talin- β1A interaction is high (approximately 450

μM, though estimation may not be accurate since as the binding curve did not reach saturation). This result conflicts with some previous studies that reported K_D of talin-β1A interaction to be less than 100 nM using surface plasmon resonance and pulldown assays (133,139). However, solution NMR was used in some studies to estimate the K_D of talin-β1A interaction to be ~500 μM (103,121,140). The variability of affinities may arise from different technologies, different talin fragments, differences in β integrin sequence used, different buffer condition and sample preparations. For example, one structure study suggests that β1D, a splice variant from β1A, binds talin with higher affinity (over 10 fold) due to two amino acids difference in sequence (140). However, despite the big discrepancies in affinity measurements, these studies suggest that NPXY tyrosine mutation or phosphorylation greatly affect talin binding. Aside from inhibiting talin binding, Abl2 mediated phosphorylation of β1 membrane proximal NPXY Tyr783 may also promote the binding of other integrin inhibitor like Dok1(102). Further experiments are required to determine the role of Abl2 mediated phosphorylation of integrin β1 on talin binding.

What is the biological importance of Abl2 mediated inhibition of talin-integrin binding?

Integrin activation by talin is essential for normal development because it regulates cell adhesion and migration, and connects the extracellular matrix with the intracellular cytoskeleton (108,116,133,139). Loss of ubiquitously expressed

integrin β1 or talin1 results in embryonic lethality (128). However, integrin inactivating proteins are also crucial for regulating integrin function and finetuning the balance between integrin activation and inactivation during many cellular processes (122-128). The most common mechanism of integrin inactivation involves binding of integrin inactivators to the integrin cytoplasmic tail, and inhibit the binding of integrin activator such as talin (122-128). Upon PDGF stimulation, Abl2 binds to integrin β1 cytoplasmic tail, and inhibits talin biding. Therefore, it is expected that Abl2 mediated inhibition of talin-integrin binding upon PDGF stimulation would result in integrin inactivation.

We assessed the activation state of integrin α5β1 by mearing the binding of fibronectin fragment to activated integrin in FACS based assay. Results show that there is no significant effect on integrin activation after PDGF stimulation or change of Abl2 expression level. Even though it is unexpected, this result can be explained with the following postulations: FRET-FLIM results demonstrated that there is only a very small decrease of FRET between talin in integrin β1 after Abl2 overexpression. The reduction of FRET is only substantial after PDGF stimulation (Fig 3.2D). Therefore, it is possible that overexpression of Abl2 in its inactivated form will not be able to affect talin-integrin binding. The observation that overexpression of Abl2 alone did not result in high level of integrin Y783 phosphorylation (Fig 3.3B) is consistent with this hypothesis. My integrin activation assay showed that PDGF stimulation did not result in any significant changes of integrin activity (Fig 3.5B) likely due to the time-sensitive nature of PDGF stimulation. Tyrosine phosphorylation level of PDGFR peaked at ~10

minutes post-PDGF stimulation, and quickly diminished due to PDGFR down regulation, a phenomenon usually involved in ligand induced receptor internalization, ubiquitin-mediated proteolysis, and dephosphorylation by phosphotyrosine phosphatase (141-143). PDGF-mediated activation of phosphatidylinositol 3 kinase (PI3K) also peaked at ~10 minutes and diminished to basal levels at ~30 minutes (144). Therefore, it is possible that the effect of PDGFR/Abl2-mediated inhibition of talin-integrin interaction is not sustained when the integrin activation assay was performed. The FACS based integrin activation assay is limited by its long preparation time. If possible, using real-time microscopy to assess integrin activation after PDGF stimulation will be more informative.

CHAPTER 4- MATERIALS AND METHODS

Molecular Cloning and Recombinant Protein Purification

Full-length Abl2 (residues 74-1182), Abl2 N-terminus (residue 74-557), Abl2 kinase domain (residue 288-539) and PDGFRβ cytoplasmic domain (residues 554-1106) were cloned with a N-terminal 6XHis tag into the pFastBac1 vector (Invitrogen), as previously described (9). Abl2 C-terminus (residue 557-1182) was cloned with an N-terminal maltose binding protein (MBP) tag into the pFastBac1 vector (Invitrogen), as previously described (35). All Abl2 and PDGFRβ point mutants were generated using PCR-based mutagenesis and confirmed by DNA sequencing. Recombinant baculoviruses expressing these constructs were generated using the Bac-to-Bac expression system (Thermo Fisher) in Sf9 insect cells, as described previously (9). After expression in Hi5 insect cells for 48 h, cells were lysed in Hi5 lysis buffer (50 mM Hepes pH 7.25, 150 mM NaCl, 5% glycerol, 20 mM imidazole, 1 mM DTT, and protease inhibitors (benzamidine, aprotinin, leupeptin, chymostatin, pepstatin A, and phenylmethylsulfonyl fluoride)). All 6XHis-tagged proteins were affinity-purified on nitrilotriacetic acid resin (Qiagen) and eluted with 250 mM imidazole. Proteins were further purified by S200 gel filtration chromatography. MBP-tagged proteins were affinity purified on amylose resin (New England Biolabs), eluted with 10 mM maltose and further purified by S200 gel filtration chromatography. All proteins were buffer-exchanged into assay buffer containing 50 mM Hepes pH 7.25, 150

mM NaCl, 5% glycerol, and 1 mM DTT using 10 ml columns packed with Sephadex G25 resin.

The Abl2 SH3-SH2 and SH2 domains were cloned in frame with GST into the pGEX-6P-1 vector and GST-Abl2-SH3-SH2 and GST-Abl2-SH2 fusion proteins were purified from BL21 (DE3) E. coli cells (Millipore Sigma) on glutathione 4B beads (GE Healthcare). The GST tags were cleaved using PreScission protease (GE Healthcare), as previously described (9). GST-CrkII was cloned into pGEX-4T-1 and purified from E. coli on glutathione 4B beads (GE Healthcare), as previously described (26). Before use in assays, all proteins were buffer-exchanged into assay buffer containing 50 mM Hepes pH 7.25, 150 mM NaCl, 5% glycerol and 1 mM DTT 10 ml columns packed with Sephadex G25 resin.

Cross-linking of Recombinant Proteins to Beads

minoLink (Thermo Scientific) beads were used to covalently link Abl2 SH2 domain following purification (9). Briefly, proteins were gently rotated with AminoLink beads overnight. 50 mM Sodium cyanoborohydride was added to catalyze the reaction. Protein was linked at a final reaction concentration of 1 μM, and the remaining reactive sites on protein-linked beads were blocked with 1 M Tris-HCl, pH 8.0 and 100 mg/ml BSA, washed, and stored in assay buffer.

Binding Assays

Binding assays were conducted as previously described (44). For determination of the PDGFRβ cytoplasmic domain (CD)-Abl2 SH2 domain binding interface, purified Abl2 SH2 and SH2 (R198K) were covalently linked to AminoLink beads

as described above and added to binding reactions at a final concentration of 1 μM. For determination of K_d values, an increasing concentration gradient of PDGFRβ CD constructs from 0 to 2 μM were used. Binding reactions were incubated for 1 h at 4°C before washing and resuspending in Laemmli sample buffer (LSB). Bead-associated material were boiled and separated on SDS PAGE gels. Gel bands were resolved with Coomassie Blue Silver stain and densities were quantified using Image J (NIH) (145). For measurements of K, band densities were plotted against concentration of the free solution protein, and binding isotherms were set using GraphPad software using the one-site specific binding equation, $Y = B_{max} * X/(K_d + X)$, where Y is specific binding, X is the concentration of the ligand, B_{max} is the maximum specific binding, in the same units as Y, and K_d is the binding affinity in the same units as X.

In Vitro *Kinase Assays*

Kinase assays were performed by preincubating 5 nM PDGFRβ CD and 0-2 μM Abl2 constructs in 50 mM Hepes pH 7.25, 150 mM NaCl, 5% glycerol, 5 mM MgCl2, 5 mM $MnCl_2$, 1 mM sodium pervanadate, 1 mM DTT for 5 min at 32°C before initiating reactions with 5 μM ATP with 0.75 μCi of [γ-^{32}P]ATP for 10 min before terminating with LSB, running on gels, and exposing to a phosphorimaging screen. Screens were scanned using a Personal Molecular Imager (Bio-Rad), and band densities were quantified using ImageJ software (145).

For *in vitro* Abl2 activation experiments, 1 μM purified recombinant Abl2 constructs were preincubated with 1 nM PDGFRβ CD for 2 hours at 32°C in 25 mM Hepes pH 7.25, 100 mM NaCl, 5% glycerol, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM sodium pervanadate, 1 mM DTT and 10 μM cold ATP. After 2 hours preincubation on ice, 25 μl reactions were initiated by addition of GST-CrkII (0-16 μM as substrate), 1 nM of preincubated Abl2 kinase proteins, 5 μM ATP, and 0.5 μCi of [γ-^{32}P] ATP. All reactions were quenched with 1X LSB after 10 mins, boiled, and separated on 10% SDS PAGE gels. Gels were stained with Blue Silver G-250 Coomassie for 30 min to visualize GST-CrkII protein bands. Bands were cut out, along with background regions within the same lane and scintillation counted along with a 1 μL sample from the kinase assay. The number of counts per minute was calculated and K_M and k_{cat} values were determined as previously (44,46).

Cell culture, construct transfection and Antibody

Experiments were performed in HEK293 cells (ATCC, Manassas, VA) and mycoplasma free WT mouse 3T3 fibroblast cells. Cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. *abl2−/−* and *abl1−/−abl2−/−* 3T3 fibroblasts were generated using CRISPR/Cas9. A guide sequence of 5'-CATGTAAAGTAACACGACGG-3' with an adjacent protospacer adjacent motif, PAM (CGG) targeting the seventh exon of Abl2 was inserted into lentiCRISPRv2 plasmid (Addgene Plasmid #52961), then transfected into HEK293T cells to generate Abl2sg1 lentivirus. WT mouse 3T3 fibroblast cells were infected with

the generated Abl2sg1 lentivirus then selected with 2 μg/ml puromycin for 72 h to generate *abl2−/−* 3T3 cells. Another guide sequence of 5'-GTTAGTTCACCATCACTCCA-3' with an adjacent protospacer adjacent motif, PAM (CGG) targeting the fourth exon of Abl1 was inserted into lentiCRISPRv2 neo plasmid (Addgene Plasmid #98292), then transfected into HEK293T cells to generate Abl1sg1 lentivirus. WT mouse 3T3 fibroblast cells were infected with the generated Abl2sg1 lentivirus and Abl1sg1 lentivirus simultaneously then selected with 2 μg/ml puromycin and 800 μg/ml G418 to generate *abl1−/−abl2−/−* DKO 3T3 cells.

The following antibodies were used for this study: phosphotyrosine (4G10; Upstate/Millipore, Billerica, MA, or affinity-purified from hybridomas), (P)-Y751 PDGFRβ (Cell Signaling, Danvers, MA), CrkII (Cell Signaling, Danvers, MA), (P)-Y221 CrkII (Cell Signaling, Danvers, MA), Abl2 (Ar11, Ar19; purified from hybridomas), HA (12CA5 purified from hybridomas), PY (4G10 purified from hybridomas), PR4, a rabbit polyclonal antiserum recognizing the C-terminal 13 amino acids of the human PDGFRβ was a generous gift from Daniel DiMaio (Yale University, New Haven, CT)

In vivo PDGFRβ pulldown binding assay

HEK293 cells were transiently transfected with WT or mutant full length PDGFRβ using polyethylenimine transfection. 48 h after transfection, cells were serum starved overnight with DMEM only medium. PDGFRβ was then stimulated with 100 nM of PDGF-BB for 10 mins. Cells were lysed in RIPA buffer (50mM Hepes, pH7.25, 150 mM NaCl,1% Nonidet P-40, 1 mM EDTA, 1% deoxycholic acid,

0.1% SDS, 0.5 mM sodium pervanadate and protease inhibitor). 500 µg of lysate were incubated with 1 µM of Abl2 SH2-linked beads in a 500 µl reaction overnight before washing and resuspending in LSB. Pulldown products were boiled, separated by SDS-PAGE, transferred, and then immunoblotted for PDGFRβ.

Immunoprecipitation

Abl2-HA was immunoprecipitated from HEK298 cells lysed in Triton lysis buffer (25 mM Hepes, pH 7.25, 150 mM NaCl, 1 mM EDTA,10% glycerol, 1% Triton X-100 0.5 mM sodium pervanadate and protease inhibitor). Cell lysate (0.5 mg; standardized to 1 mg/ml) was precleared with 20 µl of Protein A/G Plus Agarose bead (Thermo Scientific) for 1 hour at 4°C. The precleared supernatant was incubated with 20 µl of beads that had been incubated overnight with anti-HA antibody (12CA5) for 1 h at 4°C. Immunoprecipitates were washed three times with 0.5 ml of lysis buffer, suspended in 40 µl of LSB, and separated by SDS-PAGE for immunoblot analysis.

Measurement of CrkII phosphorylation

WT and *abl1−/−abl2−/−* DKO KO mouse 3T3 fibroblast were serum starved overnight before stimulation with 100 nM of PDGF-BB for 10 mins. Cells were lysed in RIPA buffer (50mM Hepes, pH 7.25, 150 mM NaCl,1% Nonidet P-40, 1 mM EDTA, 1% deoxycholic acid, 0.1% SDS, 0.5mM sodium pervanadate and protease inhibitor). 40 µg of lysate and 10 µl of Laemmli sample buffer (LSB) were boiled, separated by on SDS-PAGE, transferred, and then blotted for CrkII,

(P)-Y221 CrkII, PDGFRβ, (P)-Y751 PDGFRβ. 500 µg of cell lysate were precleared with A/G-agarose beads (Pierce Protein Biology) and incubated overnight at 4°C with Ar11 antibody beads to immunoprecipitate Abl2. Immunocomplexes were incubated with protein A/G-agarose beads for 1 h at 4°C before spinning down, washing and resuspending in LSB). Pull down products were boiled, separated by SDS-PAGE, transferred, and then immunoblotted for Abl2 and phospho-tyrosine.

In Solution Proteolysis and Phosphopeptide Enrichment

In vitro phosphorylated Abl2 (50 mg) was reduced with DTT (10 mM, 30 min, 56°C), alkylated with iodoacetamide (IAM, 30 mM, room temperature, 45 min in dark) and then digested with trypsin in a trypsin to protein ratio of 1:20 at 37°C overnight. The reaction was quenched by 1% formic acid (FA, final concentration). The resulting peptides were dried using a SpeedVac (Thermo, SPD1010), desalted with PierceTM C18 tips (Thermo), and the phosphopeptides were enriched by TitansphereTM Phos-TiO Kit (GL Science) according to the manufacturer's protocol.

LC-MS/MS Analysis

The enriched phosphopeptides were analyzed by mass spectrometry (MS) using a Dionex Ultimate 3000 nano-UHPLC system LC coupled with a Thermo Scientific Orbitrap Velos Pro mass spectrometer. The Dionex Ultimate 3000 system was equipped with an Acclaim PepMap 100 (C18, 5 µm, 100 Å, 100 µm x 2 cm, Thermo Scientific, Waltham, MA) trap column and an Acclaim PepMap

RSLC (C18, 2 µm, 100 Å, 75 µm x 50 cm, Thermo Scientific, Waltham, MA) analytical column. The phosphopeptides were separated using the following chromatography conditions: flow rate 300 nL/min, mobile phase A: 95% H_2O, 5% acetonitrile (ACN), and 0.1% FA; mobile phase B: 20% H_2O, 80% ACN, and 0.1% FA; and, linear gradient starting at 5% B at 0 min to 40% B at 105 min. The MS spray voltage was set in 2.1 kV, temperature of the heated capillary was 200°C. The MS analysis was performed using a top-10 data dependent analysis in the positive ion mode with dynamc exclusion option enabled for 30 sec. MS/MS spectra were collected using high-energy collisional dissociation (HCD) and the overall method was setup in XCalibur 2.1 software (Thermo Scientific, Waltham, MA). Raw MS and MS/MS data were then searched against a custom made database which included the sequence of recombinant Abl2 in the background of *E. coli* database, using Sequest HT algorithm within the Proteome Discoverer v2.2 (Thermo Scientific) and with the following parameters: parent mass error tolerance, 10 ppm; fragment mass error tolerance, 0.6 Da (monoisotopic); maximum missed cleavage sites, 2; variable modifications: +15.995 Da (oxidation) on methionine, and +79.996 Da (phosphorylation) on serine, threonine, and tyrosine; fixed modification: +57.021 Da (carbamidomethylation) on cysteine.

FRET-FLIM studies

FRET-FLIM studies were performed by Dr. Maddy Parsons as described in (146). β1-/- MEFs were transfected with β1-GFP WT or mutant constructs and Abl2 mRFP for binding studies, or β1-GFP WT and talin-RFP or kindlin-RFP, with

or without Arg-HA DNA for competition assays. Cells were serum starved and then treated with 30 ng/mL PDGF for 10 mins before fixing, staining, and imaging.

Integrin activation assay

The activation state of endogenous α5β1 was assessed by measuring the binding of a recombinant soluble integrin-binding fragment of fibronectin (FN9–11) in flow cytometric assays as described in (133). In experiments on CHO cells the α5β1 integrin expression was assessed in parallel by staining with PB1. Briefly, CHO were transfected with the indicated cDNAs using Lipofectamine (Invitrogen) and 24 h later cells were suspended and incubated with biotinylated recombinant FN9–11 in the presence or absence of integrin activators or inhibitors. Cells were washed and bound FN9–11 was detected with APC-conjugated streptavidin. FN9–11 binding was assessed on a FACSCalibur instrument (BD Biosciences).

Reference

1. Abelson, H. T., and Rabstein, L. S. (1970) Influence of prednisolone on Moloney leukemogenic virus in BALB-c mice. *Cancer Res* **30**, 2208-2212
2. Goff, S. P., Gilboa, E., Witte, O. N., and Baltimore, D. (1980) Structure of the Abelson murine leukemia virus genome and the homologous cellular gene: studies with cloned viral DNA. *Cell* **22**, 777-785
3. Ben-Neriah, Y., Daley, G. Q., Mes-Masson, A. M., Witte, O. N., and Baltimore, D. (1986) The chronic myelogenous leukemia-specific P210 protein is the product of the bcr/abl hybrid gene. *Science* **233**, 212-214

4. Srinivasan, D., and Plattner, R. (2006) Activation of Abl tyrosine kinases promotes invasion of aggressive breast cancer cells. *Cancer Research* **66**, 5648-5655
5. Ganguly, S. S., and Plattner, R. (2012) Activation of abl family kinases in solid tumors. *Genes Cancer* **3**, 414-425
6. Khatri, A., Wang, J., and Pendergast, A. M. (2016) Multifunctional Abl kinases in health and disease. *Journal of Cell Science* **129**, 9-16
7. Mader, C. C., Oser, M., Magalhaes, M. A., Bravo-Cordero, J. J., Condeelis, J., Koleske, A. J., and Gil-Henn, H. (2011) An EGFR-Src-Arg-cortactin pathway mediates functional maturation of invadopodia and breast cancer cell invasion. *Cancer Res* **71**, 1730-1741
8. Moresco, E. M., and Koleske, A. J. (2003) Regulation of neuronal morphogenesis and synaptic function by Abl family kinases. *Curr Opin Neurobiol* **13**, 535-544
9. Schlatterer, S. D., Acker, C. M., and Davies, P. (2011) c-Abl in neurodegenerative disease. *J Mol Neurosci* **45**, 445-452
10. Backert, S., Feller, S. M., and Wessler, S. (2008) Emerging roles of Abl family tyrosine kinases in microbial pathogenesis. *Trends Biochem Sci* **33**, 80-90
11. Wessler, S., and Backert, S. (2011) Abl family of tyrosine kinases and microbial pathogenesis. *Int Rev Cell Mol Biol* **286**, 271-300
12. Wetzel, D. M., Rhodes, E. L., Li, S., McMahon-Pratt, D., and Koleske, A. J. (2016) The Src kinases Hck, Fgr and Lyn activate Arg to facilitate IgG-mediated phagocytosis and Leishmania infection. *J Cell Sci* **129**, 3130-3143
13. 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
14. Zipfel, P. A., Zhang, W. G., Quiroz, M., and Pendergast, A. M. (2004) Requirement for Abl kinases in T cell receptor signaling. *Curr Biol* **14**, 1222-1231
15. Tanos, B., and Pendergast, A. M. (2006) Abl tyrosine kinase regulates endocytosis of the epidermal growth factor receptor. *J Biol Chem* **281**, 32714-32723
16. Tanos, B. E., and Pendergast, A. M. (2007) Abi-1 forms an epidermal growth factor-inducible complex with Cbl: role in receptor endocytosis. *Cell Signal* **19**, 1602-1609
17. Jacob, M., Todd, L. A., Majumdar, R. S., Li, Y., Yamamoto, K., and Pure, E. (2009) Endogenous cAbl regulates receptor endocytosis. *Cell Signal* **21**, 1308-1316
18. Lapetina, S., Mader, C. C., Machida, K., Mayer, B. J., and Koleske, A. J. (2009) Arg interacts with cortactin to promote adhesion-dependent cell edge protrusion. *J Cell Biol* **185**, 503-519
19. Peacock, J. G., Couch, B. A., and Koleske, A. J. (2010) The Abl and Arg Non-receptor Tyrosine Kinases Regulate Different Zones of Stress Fiber, Focal Adhesion, and Contractile Network Localization in Spreading Fibroblasts. *Cytoskeleton* **67**, 666-675
20. Qiu, Z. Z., Cang, Y., and Goff, S. P. (2010) c-Abl tyrosine kinase regulates cardiac growth and development. *P Natl Acad Sci USA* **107**, 1136-1141
21. Maiani, E., Diederich, M., and Gonfloni, S. (2011) DNA damage response: the emerging role of c-Abl as a regulatory switch? *Biochem Pharmacol* **82**, 1269-1276
22. Meltser, V., Ben-Yehoyada, M., and Shaul, Y. (2011) c-Abl tyrosine kinase in the DNA damage response: cell death and more. *Cell Death Differ* **18**, 2-4
23. Greuber, E. K., Smith-Pearson, P., Wang, J., and Pendergast, A. M. (2013) Role of ABL family kinases in cancer: from leukaemia to solid tumours. *Nat Rev Cancer* **13**, 559-571
24. Colicelli, J. (2010) ABL tyrosine kinases: evolution of function, regulation, and specificity. *Sci Signal* **3**, re6

25. Bradley, W. D., and Koleske, A. J. (2009) Regulation of cell migration and morphogenesis by Abl-family kinases: emerging mechanisms and physiological contexts. *Journal of Cell Science* **122**, 3441-3454
26. Kruh, G. D., Perego, R., Miki, T., and Aaronson, S. A. (1990) The complete coding sequence of arg defines the Abelson subfamily of cytoplasmic tyrosine kinases. *Proc Natl Acad Sci U S A* **87**, 5802-5806
27. Nagar, B., Hantschel, O., Young, M. A., Scheffzek, K., Veach, D., Bornmann, V., Clarkson, B., Superti-Furga, G., and Kuriyan, J. (2003) Structural basis for the autoinhibition of c-Abl tyrosine kinase. *Cell* **112**, 859-871
28. David-Cordonnier, M. H., Hamdane, M., Bailly, C., and D'Halluin, J. C. (1998) Determination of the human c-Abl consensus DNA binding site. *FEBS Lett* **424**, 177-182
29. Miller, A. L., Wang, Y., Mooseker, M. S., and Koleske, A. J. (2004) The Abl-related gene (Arg) requires its F-actin-microtubule cross-linking activity to regulate lamellipodial dynamics during fibroblast adhesion. *J Cell Biol* **165**, 407-419
30. Hu, Y. H., Lyu, W. Q., Lowery, L. A., and Koleske, A. J. (2019) Regulation of MT dynamics via direct binding of an Abl family kinase. *J Cell Biol* **218**, 3986-3997
31. Zhang, K., Lyu, W. Q., Yu, J., and Koleske, A. J. (2018) Abl2 is recruited to ventral actin waves through cytoskeletal interactions to promote lamellipodium extension. *Mol Biol Cell* **29**, 2863-2873
32. Cicchetti, P., Mayer, B., Thiel, G., and Baltimore, D. (1992) Identification of a protein that binds to the SH3 region of Abl and is similar to Bcr and GAP-rho. *Science* **257**, 803-806
33. Mayer, B. J., and Baltimore, D. (1994) Mutagenic analysis of the roles of SH2 and SH3 domains in regulation of the Abl tyrosine kinase. *Mol Cell Biol* **14**, 2883-2894
34. Barila, D., and Superti-Furga, G. (1998) An intramolecular SH3-domain interaction regulates c-Abl activity. *Nat Genet* **18**, 280-282
35. Pisabarro, M. T., Serrano, L., and Wilmanns, M. (1998) Crystal structure of the abl-SH3 domain complexed with a designed high-affinity peptide ligand: implications for SH3-ligand interactions. *J Mol Biol* **281**, 513-521
36. Musacchio, A., Saraste, M., and Wilmanns, M. (1994) High-Resolution Crystal-Structures of Tyrosine Kinase Sh3 Domains Complexed with Proline-Rich Peptides. *Nat Struct Biol* **1**, 546-551
37. Pendergast, A. M. (2002) The Abl family kinases: Mechanisms of regulation and signaling. *Adv Cancer Res* **85**, 51-100
38. Wagner, M. J., Stacey, M. M., Liu, B. A., and Pawson, T. (2013) Molecular Mechanisms of SH2-and PTB-Domain-Containing Proteins in Receptor Tyrosine Kinase Signaling. *Csh Perspect Biol* **5**
39. Liu, B. A., and Machida, K. (2017) Introduction: History of SH2 Domains and Their Applications. in *SH2 Domains: Methods and Protocols* (Machida, K., and Liu, B. A. eds.), Springer New York, New York, NY. pp 3-35
40. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., and et al. (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**, 767-778
41. Tinti, M., Kiemer, L., Costa, S., Miller, M. L., Sacco, F., Olsen, J. V., Carducci, M., Paoluzi, S., Langone, F., Workman, C. T., Blom, N., Machida, K., Thompson, C. M., Schutkowski, M., Brunak, S., Mann, M., Mayer, B. J., Castagnoli, L., and Cesareni, G. (2013) The SH2 domain interaction landscape. *Cell Rep* **3**, 1293-1305
42. Gifford, S. M., Liu, W. Z., Mader, C. C., Halo, T. L., Machida, K., Boggon, T. J., and Koleske, A. J. (2014) Two Amino Acid Residues Confer Different Binding Affinities of Abelson

Family Kinase Src Homology 2 Domains for Phosphorylated Cortactin. *Journal of Biological Chemistry* **289**, 19704-19713
43. Hantschel, O., and Superti-Furga, G. (2006) Mechanisms of Activation of Abl Family Kinases. in *Abl Family Kinases in Development and Disease*, Springer New York, New York, NY. pp 1-10
44. Simpson, M. A., Bradley, W. D., Harburger, D., Parsons, M., Calderwood, D. A., and Koleske, A. J. (2015) Direct interactions with the integrin beta1 cytoplasmic tail activate the Abl2/Arg kinase. *J Biol Chem* **290**, 8360-8372
45. 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
46. 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
47. Plattner, R., Koleske, A. J., Kazlauskas, A., and Pendergast, A. M. (2004) Bidirectional signaling links the Abelson kinases to the platelet-derived growth factor receptor. *Mol Cell Biol* **24**, 2573-2583
48. Boyle, S. N., Michaud, G. A., Schweitzer, B., Predki, P. F., and Koleske, A. J. (2007) A critical role for cortactin phosphorylation by Abl-family kinases in PDGF-induced dorsal-wave formation. *Curr Biol* **17**, 445-451
49. Kain, K. H., and Klemke, R. L. (2001) Inhibition of cell migration by Abl family tyrosine kinases through uncoupling of Crk-CAS complexes. *Journal of Biological Chemistry* **276**, 16185-16192
50. Hernandez, S. E., Settleman, J., and Koleske, A. J. (2004) Adhesion-dependent regulation of p190RhoGAP in the developing brain by the Abl-related gene tyrosine kinase. *Curr Biol* **14**, 691-696
51. Wong, S., and Witte, O. N. (2004) The BCR-ABL story: Bench to bedside and back. *Annu Rev Immunol* **22**, 247-306
52. Ganguly, S. S., Fiore, L. S., Sims, J. T., Friend, J. W., Srinivasan, D., Thacker, M. A., Cibull, M. L., Wang, C., Novak, M., Kaetzel, D. M., and Plattner, R. (2012) c-Abl and Arg are activated in human primary melanomas, promote melanoma cell invasion via distinct pathways, and drive metastatic progression. *Oncogene* **31**, 1804-1816
53. Advani, A. S., and Pendergast, A. M. (2002) Bcr-Abl variants: biological and clinical aspects. *Leuk Res* **26**, 713-720
54. Hantschel, O. (2012) Structure, regulation, signaling, and targeting of abl kinases in cancer. *Genes Cancer* **3**, 436-446
55. Lugo, T. G., Pendergast, A. M., Muller, A. J., and Witte, O. N. (1990) Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. *Science* **247**, 1079-1082
56. Salesse, S., and Verfaillie, C. M. (2002) BCR/ABL: from molecular mechanisms of leukemia induction to treatment of chronic myelogenous leukemia. *Oncogene* **21**, 8547-8559
57. Smith, K. M., Yacobi, R., and Van Etten, R. A. (2003) Autoinhibition of Bcr-Abl through Its SH3 Domain. *Mol Cell* **12**, 27-37
58. Mcwhirter, J. R., Galasso, D. L., and Wang, J. Y. J. (1993) A Coiled-Coil Oligomerization Domain of Bcr Is Essential for the Transforming Function of Bcr-Abl Oncoproteins. *Mol Cell Biol* **13**, 7587-7595

59. Beissert, T., Hundertmark, A., Kaburova, V., Travaglini, L., Mian, A. A., Nervi, C., and Ruthardt, M. (2008) Targeting of the N-terminal coiled coil oligomerization interface by a helix-2 peptide inhibits unmutated and imatinib-resistant BCR/ABL. *Int J Cancer* **122**, 2744-2752
60. Mian, A. A., Oancea, C., Zhao, Z., Ottmann, O. G., and Ruthardt, M. (2009) Oligomerization inhibition, combined with allosteric inhibition, abrogates the transformation potential of T315I-positive BCR/ABL. *Leukemia* **23**, 2242-2247
61. Garcia-Gutierrez, V., and Hernandez-Boluda, J. C. (2019) Tyrosine Kinase Inhibitors Available for Chronic Myeloid Leukemia: Efficacy and Safety. *Front Oncol* **9**, 603
62. Zhang, J., Adrian, F. J., Jahnke, W., Cowan-Jacob, S. W., Li, A. G., Iacob, R. E., Sim, T., Powers, J., Dierks, C., Sun, F., Guo, G. R., Ding, Q., Okram, B., Choi, Y., Wojciechowski, A., Deng, X., Liu, G., Fendrich, G., Strauss, A., Vajpai, N., Grzesiek, S., Tuntland, T., Liu, Y., Bursulaya, B., Azam, M., Manley, P. W., Engen, J. R., Daley, G. Q., Warmuth, M., and Gray, N. S. (2010) Targeting Bcr-Abl by combining allosteric with ATP-binding-site inhibitors. *Nature* **463**, 501-506
63. Ha, B. H., Simpson, M. A., Koleske, A. J., and Boggon, T. J. (2015) Structure of the ABL2/ARG kinase in complex with dasatinib. *Acta Crystallogr F Struct Biol Commun* **71**, 443-448
64. Nagar, B., Bornmann, W. G., Pellicena, P., Schindler, T., Veach, D. R., Miller, W. T., Clarkson, B., and Kuriyan, J. (2002) Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). *Cancer Res* **62**, 4236-4243
65. Harrison, S. C. (2003) Variation on an Src-like theme. *Cell* **112**, 737-740
66. Roskoski, R., Jr. (2005) Src kinase regulation by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun* **331**, 1-14
67. Engen, J. R., Wales, T. E., Hochrein, J. M., Meyn, M. A., 3rd, Banu Ozkan, S., Bahar, I., and Smithgall, T. E. (2008) Structure and dynamic regulation of Src-family kinases. *Cell Mol Life Sci* **65**, 3058-3073
68. Hantschel, O., Nagar, B., Guettler, S., Kretzschmar, J., Dorey, K., Kuriyan, J., and Superti-Furga, G. (2003) A myristoyl/phosphotyrosine switch regulates c-Abl. *Cell* **112**, 845-857
69. Arias-Salgado, E. G., Lizano, S., Sarkar, S., Brugge, J. S., Ginsberg, M. H., and Shattil, S. J. (2003) Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc Natl Acad Sci U S A* **100**, 13298-13302
70. Franz, W. M., Berger, P., and Wang, J. Y. (1989) Deletion of an N-terminal regulatory domain of the c-abl tyrosine kinase activates its oncogenic potential. *EMBO J* **8**, 137-147
71. Nagar, B., Hantschel, O., Seeliger, M., Davies, J. M., Weiss, W. I., Superti-Furga, G., and Kuriyan, J. (2006) Organization of the SH3-SH2 unit in active and inactive forms of the c-Abl tyrosine kinase. *Mol Cell* **21**, 787-798
72. Panjarian, S., Iacob, R. E., Chen, S., Engen, J. R., and Smithgall, T. E. (2013) Structure and dynamic regulation of Abl kinases. *The Journal of biological chemistry* **288**, 5443-5450
73. Chen, S., Brier, S., Smithgall, T. E., and Engen, J. R. (2007) The Abl SH2-kinase linker naturally adopts a conformation competent for SH3 domain binding. *Protein Sci* **16**, 572-581
74. Majidi, M., Hubbs, A. E., and Lichy, J. H. (1998) Activation of extracellular signal-regulated kinase 2 by a novel Abl-binding protein, ST5. *J Biol Chem* **273**, 16608-16614
75. 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

76. Lamontanara, A. J., Georgeon, S., Tria, G., Svergun, D. I., and Hantschel, O. (2014) The SH2 domain of Abl kinases regulates kinase autophosphorylation by controlling activation loop accessibility. *Nat Commun* **5**, 5470
77. Filippakopoulos, P., Kofler, M., Hantschel, O., Gish, G. D., Grebien, F., Salah, E., Neudecker, P., Kay, L. E., Turk, B. E., Superti-Furga, G., Pawson, T., and Knapp, S. (2008) Structural coupling of SH2-kinase domains links Fes and Abl substrate recognition and kinase activation. *Cell* **134**, 793-803
78. Grebien, F., Hantschel, O., Wojcik, J., Kaupe, I., Kovacic, B., Wyrzucki, A. M., Gish, G. D., Cerny-Reiterer, S., Koide, A., Beug, H., Pawson, T., Valent, P., Koide, S., and Superti-Furga, G. (2011) Targeting the SH2-kinase interface in Bcr-Abl inhibits leukemogenesis. *Cell* **147**, 306-319
79. Dorey, K., Engen, J. R., Kretzschmar, J., Wilm, M., Neubauer, G., Schindler, T., and Superti-Furga, G. (2001) Phosphorylation and structure-based functional studies reveal a positive and a negative role for the activation loop of the c-Abl tyrosine kinase. *Oncogene* **20**, 8075-8084
80. Chen, S., O'Reilly, L. P., Smithgall, T. E., and Engen, J. R. (2008) Tyrosine phosphorylation in the SH3 domain disrupts negative regulatory interactions within the c-Abl kinase core. *J Mol Biol* **383**, 414-423
81. Merő, B., Radnai, L., Gógl, G., Tőke, O., Leveles, I., Koprivanacz, K., Szeder, B., Dülk, M., Kudlik, G., Vas, V., Cserkaszky, A., Sipeki, S., Nyitray, L., Vértessy, B. G., and Buday, L. (2019) Structural insights into the tyrosine phosphorylation-mediated inhibition of SH3 domain-ligand interactions. *The Journal of biological chemistry* **294**, 4608-4620
82. Steen, H., Fernandez, M., Ghaffari, S., Pandey, A., and Mann, M. (2003) Phosphotyrosine Mapping in Bcr/Abl Oncoprotein Using Phosphotyrosine-specific Immonium Ion Scanning*. *Molecular & Cellular Proteomics* **2**, 138-145
83. Salomon, A. R., Ficarro, S. B., Brill, L. M., Brinker, A., Phung, Q. T., Ericson, C., Sauer, K., Brock, A., Horn, D. M., Schultz, P. G., and Peters, E. C. (2003) Profiling of tyrosine phosphorylation pathways in human cells using mass spectrometry. *Proceedings of the National Academy of Sciences* **100**, 443-448
84. Hantschel, O., and Superti-Furga, G. (2004) Regulation of the c-Abl and Bcr-Abl tyrosine kinases. *Nat Rev Mol Cell Biol* **5**, 33-44
85. 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. *Gene Dev* **13**, 2400-2411
86. Plattner, R., Irvin, B. J., Guo, S. L., 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-gamma 1. *Nat Cell Biol* **5**, 309-319
87. Srinivasan, D., Kaetzel, D. M., and Plattner, R. (2009) Reciprocal regulation of Abl and receptor tyrosine kinases. *Cell Signal* **21**, 1143-1150
88. Bradley, W. D., Hernandez, S. E., Settleman, J., and Koleske, A. J. (2006) Integrin signaling through arg activates p190RhoGAP by promoting its binding to p120RasGAP and recruitment to the membrane. *Mol Biol Cell* **17**, 4827-4836
89. Shaul, Y., and Ben-Yehoyada, M. (2005) Role of c-Abl in the DNA damage stress response. *Cell Res* **15**, 33-35
90. Gu, J. J., Ryu, J. R., and Pendergast, A. M. (2009) Abl tyrosine kinases in T-cell signaling. *Immunol Rev* **228**, 170-183
91. Harburger, D. S., and Calderwood, D. A. (2009) Integrin signalling at a glance. *Journal of Cell Science* **122**, 159-163

92. Lewis, J. M., Baskaran, R., Taagepera, S., Schwartz, M. A., and Wang, J. Y. (1996) Integrin regulation of c-Abl tyrosine kinase activity and cytoplasmic-nuclear transport. *Proc Natl Acad Sci U S A* **93**, 15174-15179
93. Beaty, B. T., Sharma, V. P., Bravo-Cordero, J. J., Simpson, M. A., Eddy, R. J., Koleske, A. J., and Condeelis, J. (2013) beta1 integrin regulates Arg to promote invadopodial maturation and matrix degradation. *Mol Biol Cell* **24**, 1661-1675, S1661-1611
94. Moresco, E. M., Donaldson, S., Williamson, A., and Koleske, A. J. (2005) Integrin-mediated dendrite branch maintenance requires Abelson (Abl) family kinases. *J Neurosci* **25**, 6105-6118
95. Kerrisk, M. E., and Koleske, A. J. (2013) Arg kinase signaling in dendrite and synapse stabilization pathways: Memory, cocaine sensitivity, and stress. *Int J Biochem Cell B* **45**, 2496-2500
96. Omar, M. H., Kerrisk Campbell, M., Xiao, X., Zhong, Q., Brunken, W. J., Miner, J. H., Greer, C. A., and Koleske, A. J. (2017) CNS Neurons Deposit Laminin α5 to Stabilize Synapses. *Cell reports* **21**, 1281-1292
97. Tybulewicz, V. L. J., Crawford, C. E., Jackson, P. K., Bronson, R. T., and Mulligan, R. C. (1991) Neonatal Lethality and Lymphopenia in Mice with a Homozygous Disruption of the C-Abl Protooncogene. *Cell* **65**, 1153-1163
98. Schwartzberg, P. L., Stall, A. M., Hardin, J. D., Bowdish, K. S., Humaran, T., Boast, S., Harbison, M. L., Robertson, E. J., and Goff, S. P. (1991) Mice Homozygous for the Ablm1 Mutation Show Poor Viability and Depletion of Selected B-Cell and T-Cell Populations. *Cell* **65**, 1165-1175
99. Zipfel, P. A., Grove, M., Blackburn, K., Fujimoto, M., Tedder, T. F., and Pendergast, A. M. (2000) The c-Abl tyrosine kinase is regulated downstream of the B cell antigen receptor and interacts with CD19. *J Immunol* **165**, 6872-6879
100. Gil-Henn, H., Patsialou, A., Wang, Y., Warren, M. S., Condeelis, J. S., and Koleske, A. J. (2013) Arg/Abl2 promotes invasion and attenuates proliferation of breast cancer in vivo. *Oncogene* **32**, 2622-2630
101. Weigel, M. T., Banerjee, S., Arnedos, M., Salter, J., A'Hern, R., Dowsett, M., and Martin, L. A. (2013) Enhanced expression of the PDGFR/Abl signaling pathway in aromatase inhibitor-resistant breast cancer. *Ann Oncol* **24**, 126-133
102. Oxley, C. L., Anthis, N. J., Lowe, E. D., Vakonakis, I., Campbell, I. D., and Wegener, K. L. (2008) An integrin phosphorylation switch: the effect of beta3 integrin tail phosphorylation on Dok1 and talin binding. *J Biol Chem* **283**, 5420-5426
103. Anthis, N. J., Haling, J. R., Oxley, C. L., Memo, M., Wegener, K. L., Lim, C. J., Ginsberg, M. H., and Campbell, I. D. (2009) Beta integrin tyrosine phosphorylation is a conserved mechanism for regulating talin-induced integrin activation. *J Biol Chem* **284**, 36700-36710
104. MacGrath, S. M., and Koleske, A. J. (2012) Arg/Abl2 modulates the affinity and stoichiometry of binding of cortactin to F-actin. *Biochemistry* **51**, 6644-6653
105. Leung, K. K., Hause, R. J., Jr., Barkinge, J. L., Ciaccio, M. F., Chuu, C.-P., and Jones, R. B. (2014) Enhanced prediction of Src homology 2 (SH2) domain binding potentials using a fluorescence polarization-derived c-Met, c-Kit, ErbB, and androgen receptor interactome. *Mol Cell Proteomics* **13**, 1705-1723
106. Roumiantsev, S., Shah, N. P., Gorre, M. E., Nicoll, J., Brasher, B. B., Sawyers, C. L., and Van Etten, R. A. (2002) Clinical resistance to the kinase inhibitor STI-571 in chronic myeloid leukemia by mutation of Tyr-253 in the Abl kinase domain P-loop. *Proc Natl Acad Sci U S A* **99**, 10700-10705

107. Azam, M., Latek, R. R., and Daley, G. Q. (2003) Mechanisms of autoinhibition and STI-571/imatinib resistance revealed by mutagenesis of BCR-ABL. *Cell* **112**, 831-843
108. Calderwood, D. A. (2004) Integrin activation. *J Cell Sci* **117**, 657-666
109. Woodard, A. S., Garcia-Cardena, G., Leong, M., Madri, J. A., Sessa, W. C., and Languino, L. R. (1998) The synergistic activity of alphavbeta3 integrin and PDGF receptor increases cell migration. *J Cell Sci* **111 (Pt 4)**, 469-478
110. Eliceiri, B. P. (2001) Integrin and growth factor receptor crosstalk. *Circ Res* **89**, 1104-1110
111. Schwartz, M. A., and Ginsberg, M. H. (2002) Networks and crosstalk: integrin signalling spreads. *Nat Cell Biol* **4**, E65-68
112. Edick, M. J., Tesfay, L., Lamb, L. E., Knudsen, B. S., and Miranti, C. K. (2007) Inhibition of integrin-mediated crosstalk with epidermal growth factor receptor/Erk or Src signaling pathways in autophagic prostate epithelial cells induces caspase-independent death. *Mol Biol Cell* **18**, 2481-2490
113. Takada, Y., Takada, Y. K., and Fujita, M. (2017) Crosstalk between insulin-like growth factor (IGF) receptor and integrins through direct integrin binding to IGF1. *Cytokine Growth Factor Rev* **34**, 67-72
114. Zemskov, E. A., Loukinova, E., Mikhailenko, I., Coleman, R. A., Strickland, D. K., and Belkin, A. M. (2009) Regulation of platelet-derived growth factor receptor function by integrin-associated cell surface transglutaminase. *J Biol Chem* **284**, 16693-16703
115. Warren, M. S., Bradley, W. D., Gourley, S. L., Lin, Y. C., Simpson, M. A., Reichardt, L. F., Greer, C. A., Taylor, J. R., and Koleske, A. J. (2012) Integrin beta 1 Signals through Arg to Regulate Postnatal Dendritic Arborization, Synapse Density, and Behavior. *Journal of Neuroscience* **32**, 2824-2834
116. Tadokoro, S., Shattil, S. J., Eto, K., Tai, V., Liddington, R. C., de Pereda, J. M., Ginsberg, M. H., and Calderwood, D. A. (2003) Talin binding to integrin beta tails: a final common step in integrin activation. *Science* **302**, 103-106
117. Morse, E. M., Brahme, N. N., and Calderwood, D. A. (2014) Integrin cytoplasmic tail interactions. *Biochemistry* **53**, 810-820
118. Garcia-Alvarez, B., de Pereda, J. M., Calderwood, D. A., Ulmer, T. S., Critchley, D., Campbell, I. D., Ginsberg, M. H., and Liddington, R. C. (2003) Structural determinants of integrin recognition by talin. *Mol Cell* **11**, 49-58
119. Wegener, K. L., Partridge, A. W., Han, J., Pickford, A. R., Liddington, R. C., Ginsberg, M. H., and Campbell, I. D. (2007) Structural basis of integrin activation by talin. *Cell* **128**, 171-182
120. Goksoy, E., Ma, Y. Q., Wang, X., Kong, X., Perera, D., Plow, E. F., and Qin, J. (2008) Structural basis for the autoinhibition of talin in regulating integrin activation. *Mol Cell* **31**, 124-133
121. Anthis, N. J., Wegener, K. L., Ye, F., Kim, C., Goult, B. T., Lowe, E. D., Vakonakis, I., Bate, N., Critchley, D. R., Ginsberg, M. H., and Campbell, I. D. (2009) The structure of an integrin/talin complex reveals the basis of inside-out signal transduction. *EMBO J* **28**, 3623-3632
122. Liu, W., Draheim, K. M., Zhang, R., Calderwood, D. A., and Boggon, T. J. (2013) Mechanism for KRIT1 release of ICAP1-mediated suppression of integrin activation. *Mol Cell* **49**, 719-729
123. Draheim, K. M., Huet-Calderwood, C., Simon, B., and Calderwood, D. A. (2017) Nuclear Localization of Integrin Cytoplasmic Domain-associated Protein-1 (ICAP1) Influences beta1 Integrin Activation and Recruits Krev/Interaction Trapped-1 (KRIT1) to the Nucleus. *J Biol Chem* **292**, 1884-1898

124. Rantala, J. K., Pouwels, J., Pellinen, T., Veltel, S., Laasola, P., Mattila, E., Potter, C. S., Duffy, T., Sundberg, J. P., Kallioniemi, O., Askari, J. A., Humphries, M. J., Parsons, M., Salmi, M., and Ivaska, J. (2011) SHARPIN is an endogenous inhibitor of β1-integrin activation. *Nat Cell Biol* **13**, 1315-1324
125. Kiema, T., Lad, Y., Jiang, P., Oxley, C. L., Baldassarre, M., Wegener, K. L., Campbell, I. D., Ylanne, J., and Calderwood, D. A. (2006) The molecular basis of filamin binding to integrins and competition with talin. *Mol Cell* **21**, 337-347
126. Legate, K. R., and Fässler, R. (2009) Mechanisms that regulate adaptor binding to β-integrin cytoplasmic tails. *Journal of Cell Science* **122**, 187-198
127. Liu, J., Das, M., Yang, J., Ithychanda, S. S., Yakubenko, V. P., Plow, E. F., and Qin, J. (2015) Structural mechanism of integrin inactivation by filamin. *Nat Struct Mol Biol* **22**, 383-389
128. Bouvard, D., Pouwels, J., De Franceschi, N., and Ivaska, J. (2013) Integrin inactivators: balancing cellular functions in vitro and in vivo. *Nat Rev Mol Cell Biol* **14**, 430-442
129. Veevers-Lowe, J., Ball, S. G., Shuttleworth, A., and Kielty, C. M. (2011) Mesenchymal stem cell migration is regulated by fibronectin through α5β1-integrin-mediated activation of PDGFR-β and potentiation of growth factor signals. *Journal of Cell Science* **124**, 1288-1300
130. Primac, I., Maquoi, E., Blacher, S., Heljasvaara, R., Van Deun, J., Smeland, H. Y., Canale, A., Louis, T., Stuhr, L., Sounni, N. E., Cataldo, D., Pihlajaniemi, T., Pequeux, C., De Wever, O., Gullberg, D., and Noel, A. (2019) Stromal integrin α11 regulates PDGFR-β signaling and promotes breast cancer progression. *J Clin Invest* **129**, 4609-4628
131. Li, H., Deng, Y., Sun, K., Yang, H., Liu, J., Wang, M., Zhang, Z., Lin, J., Wu, C., Wei, Z., and Yu, C. (2017) Structural basis of kindlin-mediated integrin recognition and activation. *Proc Natl Acad Sci U S A* **114**, 9349-9354
132. Mould, A. P., Akiyama, S. K., and Humphries, M. J. (1995) Regulation of integrin alpha 5 beta 1-fibronectin interactions by divalent cations. Evidence for distinct classes of binding sites for Mn2+, Mg2+, and Ca2+. *J Biol Chem* **270**, 26270-26277
133. Bouaouina, M., Lad, Y., and Calderwood, D. A. (2008) The N-terminal domains of talin cooperate with the phosphotyrosine binding-like domain to activate beta1 and beta3 integrins. *J Biol Chem* **283**, 6118-6125
134. Soung, Y. H., Clifford, J. L., and Chung, J. (2010) Crosstalk between integrin and receptor tyrosine kinase signaling in breast carcinoma progression. *Bmb Rep* **43**, 311-318
135. Somanath, P. R., Malinin, N. L., and Byzova, T. V. (2009) Cooperation between integrin alphavbeta3 and VEGFR2 in angiogenesis. *Angiogenesis* **12**, 177-185
136. Ivaska, J., and Heino, J. (2011) Cooperation Between Integrins and Growth Factor Receptors in Signaling and Endocytosis. *Annu Rev Cell Dev Bi* **27**, 291-320
137. Meves, A., Geiger, T., Zanivan, S., DiGiovanni, J., Mann, M., and Fässler, R. (2011) β1 integrin cytoplasmic tyrosines promote skin tumorigenesis independent of their phosphorylation. *Proceedings of the National Academy of Sciences* **108**, 15213
138. Meves, A., Stremmel, C., Bottcher, R. T., and Fassler, R. (2013) beta1 integrins with individually disrupted cytoplasmic NPxY motifs are embryonic lethal but partially active in the epidermis. *J Invest Dermatol* **133**, 2722-2731
139. Calderwood, D. A., Yan, B., de Pereda, J. M., Alvarez, B. G., Fujioka, Y., Liddington, R. C., and Ginsberg, M. H. (2002) The phosphotyrosine binding-like domain of talin activates integrins. *J Biol Chem* **277**, 21749-21758
140. Anthis, N. J., Wegener, K. L., Critchley, D. R., and Campbell, I. D. (2010) Structural diversity in integrin/talin interactions. *Structure* **18**, 1654-1666

141. Chiarugi, P., Cirri, P., Taddei, M. L., Talini, D., Doria, L., Fiaschi, T., Buricchi, F., Giannoni, E., Camici, G., Raugei, G., and Ramponi, G. (2002) New perspectives in PDGF receptor downregulation: the main role of phosphotyrosine phosphatases. *J Cell Sci* **115**, 2219-2232
142. Lennartsson, J., Wardega, P., Engstrom, U., Hellman, U., and Heldin, C. H. (2006) Alix facilitates the interaction between c-Cbl and platelet-derived growth factor beta-receptor and thereby modulates receptor down-regulation. *J Biol Chem* **281**, 39152-39158
143. Cao, C., Li, Y. P., Leng, Y. M., Li, P., Ma, Q. J., and Kufe, D. (2005) Ubiquitination and degradation of the Arg tyrosine kinase is regulated by oxidative stress. *Oncogene* **24**, 2433-2440
144. Choudhury, G. G., Biswas, P., Grandaliano, G., Fouqueray, B., Harvey, S. A., and Abboud, H. E. (1994) PDGF-mediated activation of phosphatidylinositol 3 kinase in human mesangial cells. *Kidney Int* **46**, 37-47
145. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**, 671-675
146. Parsons, M., Messent, A. J., Humphries, J. D., Deakin, N. O., and Humphries, M. J. (2008) Quantification of integrin receptor agonism by fluorescence lifetime imaging. *J Cell Sci* **121**, 265-271

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