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The Abl/Abi signaling links WAVE regulatory complex to Cbl E3 ubiquitin ligase and is essential for breast cancer cell metastasis ☆

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Abstract

The family of Abelson interactor (Abi) proteins is a component of WAVE regulatory complex (WRC) and a downstream target of Abelson (Abl) tyrosine kinase. The fact that Abi proteins also interact with diverse membrane proteins and intracellular signaling molecules places these proteins at a central position in the network that controls cytoskeletal functions and cancer cell metastasis. Here, we identified a motif in Abi proteins that conforms to consensus sequences found in a cohort of receptor and non-receptor tyrosine kinases that bind to Cbl-tyrosine kinase binding domain. The phosphorylation of tyrosine 213 in this motif is essential for Abi degradation. Double knockout of c-Cbl and Cbl B in Bcr-Abl-transformed leukemic cells abolishes Abi1, Abi2, and WAVE2 degradation. Moreover, knockout of Abi1 reduces Src family kinase Lyn activation in Bcr-Abl-positive leukemic cells and promotes EGF-induced EGF receptor downregulation in breast cancer cells. Importantly, Abi1 depletion impeded breast cancer cell invasion in vitro and metastasis in mouse xenografts. Together, these studies uncover a novel mechanism by which the WRC and receptor/non-receptor tyrosine kinases are regulated and identify Abi1 as a potential therapeutic target for metastatic breast cancer.

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Keywords: Abi1, Cbl, WRC, Metastasis, Actin cytoskeleton, E3 ubiquitinligase, Proteolysis, EGFR, Src family tyrosine kinases

Introduction

Abelson (Abl) family proteins encoded by *ABL1* and *ABL2*, also known as Abelson-related gene (*ARG*), are non-receptor tyrosine kinases that integrate signals from membrane proteins to various downstream pathways that are important for many cellular and physiological functions [1,2]. Abnormal activation of Abl has been found in association with human cancers such as chronic myelogenous leukemia (CML), breast cancer, and melanoma [3–5]. Therapies that target deregulated Abl tyrosine kinases using small molecule tyrosine kinase inhibitors (TKIs), such as imatinib and nilotinib, have revolutionized the treatment of CML with remarkable rates of sustained complete cytogenetic remission and disease-free survival [6]. Despite the remarkable success of Abl-targeted therapies in the treatment of human leukemia [7,8], clinical trials using these therapies in unselected breast cancer patients and other patients with solid tumors did not achieve similar success [9]. Lack of a mechanistic insight into the role of Abl in solid tumor development as well as molecular markers for patient selection is a critical barrier to the clinical utility of Abl-targeted therapies [5].

Abl kinases exert their cellular functions in cooperation with additional cytoplasmic and nuclear effectors such as those involved in the regulation of

Abbreviations: Abi1, Abl interactor 1; Abi2, Abl interactor 2; Arp 2/3, Actin related protein 2/3; CML, chronic myelogenous leukemia; Cbl-TKB, Cbl-tyrosine kinases binding; EGFR, epidermal growth factor receptor; IL-3, interleukin 3; IM, imatinib; KO, knockout; MAPK, mitogen-activated protein kinase; PTK, protein tyrosine kinase; PI3K, Phosphoinositide 3-kinase; shRNA, short hairpin RNA; TKI, tyrosine kinase inhibitor; WRC, WAVE regulatory complex.

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mitogenic and apoptotic pathways [1,3,4]. They are also capable of binding to cellular proteins involved in the regulation of cytoskeletal functions [1,2]. Among these proteins is the family of Abl interactor (Abi) proteins [10,11]. The family of Abi proteins, consisting of Abi1, Abi2, and Abi3, is a key regulator of Rac-dependent actin polymerization [12,13]. In mammalian cells, Abi proteins form a complex with *WA*SP-family *ver*prolin-homologous (WAVE), hematopoietic stem progenitor cell 300 (Hspc 300), Nck-associated protein (Nap), and specifically Rac1-associated protein (Sra) [12,13]. This complex, named the WAVE regulatory complex (WRC), regulates the activity of the actin nucleation factor actin related protein 2/3 (Arp 2/3) complex and thereby, controls actin dynamics in the leading edge of motile cells [14]. When it exists as a complex, the nucleation promoting activity of WAVE is inhibited [12,15–17]. Protein phosphorylation or binding with small G-protein Rac through Abi and Sra may activate its nucleation promoting activity [12,15]. Molecular and genetic studies showed that as a complex WRC is stable, but depletion of one component such as Abi leads to downregulation of the complex [18]. Previously, we have shown that Abi2 undergoes a ubiquitin-dependent proteolysis in an Abl tyrosine kinase dependent fashion [19]. Tanos et.al. also reported that Abi1 interacts with ***C***asitas ***B***-lineasge ***L***ymphoma (Cbl), an E3 ubiquitin ligase that plays a key role in regulating membrane receptor endocytosis [20]. While these studies suggest an involvement of Abl tyrosine kinase and Cbl E3 ubiquitin ligase in the Abi-mediated regulation of fundamental cellular functions, the exact mechanism remains unclear.

Here, we report that Abl tyrosine kinases link Abi proteins to a Cbl-dependent proteolysis pathway by phosphorylating Abi tyrosine 213 (Y213). Depletion of Abi1 expression not only downregulates WRC and reduces the activation of Src family kinase Lyn in Bcr-Abl-positive leukemic cell lines, but also promotes epidermal growth factor (EGF) induced EGF receptor (EGFR) downregulation in triple negative breast cancer cell line MDA-MB-231. Importantly, we show that depletion of Abi1 in metastatic MDA-MB-231 cell subline LM2-4175 impairs its invasion in vitro and metastasis in vivo.

Materials and methods

Cell lines and reagents

Ba/F3 cells were grown in RPMI 1640 containing 10% fetal bovine serum (FBS) and 15% WEHI3-conditioned medium as a source of IL3. The Ba/F3 cell lines expressing $p185^{Bcr\text{-}Abl}$ and K562 cells with or without Abi1 deficiency were cultured in RPMI containing 10% FBS. MCF-7, MDA-MB-231 cells and the metastatic sublines of MDA-MB-231 cells, 231Br and LM2-4175 (a gift from Dr. Joan Massague), were grown in EMEM containing 10% FBS and 1 mM sodium pyruvate. The preparation of rabbit polyclonal antibodies against Abi1 and Abi2 has been described previously [11]. The antibodies against Abl, c-Cbl, EGFR, ubiquitin, pan-Lyn were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and the rabbit monoclonal antibodies for hemagglutinins (HA), Cbl-B, pan- and phospho-Akt (Ser 473), phospho-pSrc (Tyr 416), and WAVE2 (Thr202/Tyr204) were purchased from the Cell Signaling Technology, Inc (Danvers, MA). Antibodies against green fluorescence protein (GFP) and the protease inhibitors were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, MA). The monoclonal anti-β-actin antibody, Calpain inhibitor ALLN, lysosome inhibitor chloroquine, and the protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). The Abl inhibitor imatinib was purchased from LC Laboratories (Woburn, MA). Proteasome inhibitor MG132 and lysosome inhibitor bafilomycin A1 were purchased from the Cell Signaling Technology, Inc (Danvers, MA). Proteasome inhibitor lactacystin was purchased from AdipoGen Corporation (San Diego, CA). The rabbit polyclonal antibodies against Abi1 phospho-tyrosine 213 was generated with Proteintech Group Inc. (Chicago, IL) using the tyrosine 213 phospho-peptide VKPPTVPNDY(PO_4) MTSPARLG as antigen and was purified by affinity chromatography column.

Plasmid constructs

The construction of murine stem cell virus (MSCV) vectors expressing enhanced green fluorescence protein (EGFP)-tagged wild type and mutant forms of Abi1 proteins has been described previously [21]. We also replaced cDNA sequences encoding for EGFP in MSCV-GFP plasmid with the cDNA sequences encoding for 2X HA-tag. The resultant vector, MSCV-HA, was used to subclone the cDNAs that express HA-tagged wild type and mutant forms of Abi1 and Abi2. The cDNAs encoding Abi1Y213F and Abi2Y213F were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and human Abi1 and Abi2 cDNAs as template. The mutated cDNAs were then subcloned into MSCV-GFP and MSCV-HA at *Bgl*II/*Xho*I sites. The desired mutations were confirmed by sequencing analysis.

CRISPR/CAS9-mediated gene editing

To generate Abi1 deficient K562 and LM2-4175 cells, CRISPR/Cas9-mediated gene editing was performed as described previously [22]. Both strands of oligo DNAs encoding for two gRNAs that specifically target Abi1 exon 1 sequences (5'ACAGGGCGCTCCATATTCGC3') were designed using an online CRISPR design tool [23]. The two pairs of oligos were synthesized, annealed, and cloned into plasmid pSpCas9 (BB)-2A-Puro, a gift from Dr. Feng Zhang (Addgene plasmid #48139; http://n2t.net/addgene:48139; RRID:Addgene_48139), respectively, at the BbsI site. The resultant plasmids were amplified and transfected into K562 cells and LM2-4175 cells by electroporation and Fugene-mediated transfection. The methods described above are also used for generation of c-Cbl, Cbl-B, and Cbl/Cbl-B double knockout $p185^{Bcr\text{-}Abl}$ cells. The oligo DNAs encoding for the gRNAs that specifically target c-Cbl and Cbl-B sequences are as follows: *c-Cbl gRNA A*: 5'GCCTGTCGAAAGCTCTTCCA3'; *c-Cbl gRNA B*: 5'AGCCTTGCTGTAACTCACCC3'; *c-Cbl gRNA C*: 5'TCACTGACCTGCCAGGTTTG3'; *Cbl-B gRNA A*: 5'GCCTGTCGAAAGCTCTTCCA3'; *Cbl-B gRNA B*: 5'AGCCTTGCTGTAACTCACCC3'; *Cbl-B gRNA C*: 5'TCACTGACCTGCCAGGTTTG3'.

Indel mutations analysis of Abi1 knockout cell lines

To analyze indel mutations in *ABI1* deficient LM2-4175 cells, the genomic DNAs from the knockout clonal lines were purified using the Wizard genomic DNA purification kit (Promega, Madison, WI). Polymerization chain reaction (PCR) was then performed to amplify *ABI1* exon 1 using the genomic DNA as template and the following oligos as primers: forward 5' AGCCCTGTGGTCTGTCCTAA3' and reverse 5'ACAGGGCGCTCCATATTCGC3'. The amplified DNA was digested by restriction enzyme and cloned to plasmid pBSK and the resultant plasmids were sequenced to identify indel.

Three-dimensional (3D) spheroid invasion assay

The 3D spheroid invasion assay was performed as described previously with minor modification [24]. Briefly, reproducibly sized tumor spheroids of LM2-4175 cells with or without *ABI1* deficiency were obtained by plating 1×10^3 cells/100 μl/well cells into ultra-low attachment (ULA) 96-well round bottom plates. Four-day post-initiation spheroids were embedded into methylcellulose and collagen matrix thereby providing a semi-solid structure into which tumor cells invade and spread out of the spheroid. Spheroids were located centrally at the base of each well and invasion into the methylcellulose and collagen matrix was easily monitored at intervals starting from t = 0, 24,

48, 72, and 144 hours using Olympus IX51 inverted microscope. The images of spheroids were analyzed using Abode Photoshop software.

In vivo metastasis studies

The animal studies were performed in accordance with a protocol approved by the Institutional Animal Care Use Committee at the Texas Tech University Health Sciences Center. Female Nu/Nu mice (Charles River Laboratories, Hollister, CA) at the age of 6-week were randomly grouped (n = 5 for each group) and anesthetized with 2% isoflurane. The mice were injected with 2.5×10^5 LM2-4175 cells or knockout cells in 100 µl phosphate-buffered saline (PBS) into the left cardiac ventricle using a stereotaxic instrument (Stoelting Co., Wood Dale, IL) while control mice were intracardiacally injected with 100 µl PBS only. Mice were closely monitored over a period of 60 days and weighed weekly after injection. Bioluminescence imaging (BLI, IVIS imager, PerkinElmer, Waltham, MA) was performed after subcutaneous injection of D-luciferin (150 mg/kg; Gold Biotechnology, St Louis, MO). Total flux (photons/second) was quantified using Living Image software (PerkinElmer). Mice were sacrificed by CO_2 asphyxiation when showed excessive weight loss, became moribund, or at the end of the experiment and survival time was calculated from the time of cell inoculation to death of mice. Brain, kidney, liver, and lung were weighed and examined for tumors or other visible abnormalities. Collection of lungs was performed immediately after sacrifice and the tissues were fixed and paraffin embedded. Tissue sections were prepared and cancer metastases were confirmed by a gross pathology analysis of Haemotoxylin and Eosin (H&E) stained tissues sections.

Statistical analysis

Descriptive statistics were generated for all quantitative data with presentation of means ± SDs. Significance of comparisons between two experimental groups was tested using the Student's *t* test. The logrank test was used to determine differences of the Kaplan-Meier survival curves. $P < 0.05$ was considered statistically significant.

Results

Abl tyrosine kinase-dependent downregulation of Abi proteins in Bcr-Abl-positive leukemic cells

As we previously reported [19], the transformation of hematopoietic cells by Bcr-Abl induces a rapid ubiquitin-dependent degradation of Abi2 (Fig. 1 A). The Bcr-Abl induced degradation of Abi2 is dependent on Abl tyrosine kinase activity because the Abl tyrosine kinase inhibitor, imatinib, inhibits the Abi2 degradation in both $p185^{Bcr\text{-}Abl}$-transformed Ba/F3 cells ($p185^{Bcr\text{-}Abl}$) and $p210^{Bcr\text{-}Abl}$-positive human CML cell line K562 (Fig. 1B). In addition to Abi2, Bcr-Abl transformation also induces a down-regulation of Abi1 and WAVE2 in these cells, albeit to a lesser extent (Fig. 1B and 1C). This Abl tyrosine kinase-dependent Abi1 downregulation is at least in part due to protein degradation, as evidenced by cycloheximide chase analysis of the protein degradation in the $p185^{Bcr\text{-}Abl}$ cells expressing the HA-tagged Abi1 that were treated with or without imatinib (Fig. 1C). To determine which pathway mediates the Bcr-Abl induced Abi2 degradation, we treated $p185^{Bcr\text{-}Abl}$ cells with proteasome inhibitors MG132 and lactacystin, lysosome inhibitors bafilomycin A1 and chloroquine, as well as calpain inhibitor ALLN. As shown in Fig. 1D, lysosome inhibitors bafilomycin A1 and chloroquine failed to block Abi2 degradation in $p185^{Bcr\text{-}Abl}$ cells. In contrast, Bcr-Abl induced downregulation of Abi2 was effectively inhibited by the proteasome inhibitor MG132 and, to a lesser extent, by the proteasome inhibitor lactacystin and calpain inhibitor ALLN, which also inhibits proteasome with a Ki value of 5 μM (Fig. 1D). Thus, these data suggest that the downregulation of Abi2 in Bcr-Abl-positive leukemic cells is mediated in part by ubiquitin-dependent proteolysis.

Tyrosine 213 of Abi1 is phosphorylated and is required for Bcr-Abl-induced downregulation of Abi proteins

Previous studies have identified the tyrosine 213 (Y213) in Abi1 as a phosphorylation site of active Abl tyrosine kinases [25,26]. Using an antibody raised against a peptide flanking phosphorylated Y213 (Fig. 2 A, α-pY213), we were able to detect the Y213-phosphorylated Abi1 in $p185^{Bcr\text{-}Abl}$ transformed Ba/F3 cells (Fig. 2A, lane 2), but not parental Ba/F3 cells (Fig. 2A, lane 1). Our antibody is specific for the Y213-phosphorylated Abi1 because it did not cross-react with a GFP-tagged Abi1 mutant in which the Y213 is mutated to phenylalanine (GFP-Abi1Y213F) in $p185^{Bcr\text{-}Abl}$ transformed Ba/F3 cells (Fig. 2A, lane 4). Moreover, the treatment of $p185^{Bcr\text{-}Abl}$ transformed Ba/F3 cells with Abl kinase inhibitor imatinib abrogated the Abi1 Y213 phosphorylation (Fig. 2A, lane 3). Together, these data indicate that Y213 is a major site that is phosphorylated by $p185^{Bcr\text{-}Abl}$.

To determine if Y213 is required for Bcr-Abl-induced down regulation of Abi proteins, we generated the constructs that express either a HA-tagged wild type Abi1 (Fig. 2B, HA-Abi1wt) or a mutant form of Abi1 in which the codon that encodes for tyrosine 213 is mutated to encode for a phenylalanine instead (Fig. 2B, HA-Abi1 Y213F). After transfection into $p185^{Bcr\text{-}Abl}$ cells, we examined protein stability of the wild type Abi1 (Fig. 2B, up panel) and compared it to that of Abi1 Y213F (Fig. 2B, bottom panel). Our results show that the Abi1 Y213F mutant protein is more stable than the wild type Abi1 in $p185^{Bcr\text{-}Abl}$ cells.

We also generated the constructs that express the HA-tagged wild type Abi2 (HA-Abi2wt) and mutant Abi2 bearing Y213F mutation (HA-Abi2 Y213F). Expression of the wild type Abi2 in $p185^{Bcr\text{-}Abl}$ cells has been difficult and is barely detected (Fig. 2C, lane 1). In contrast, the construct encoding for mutant Abi1 Y213F is readily expressed (Fig. 2C, lane 2). Failure of expression of wild type Abi2 in $p185^{Bcr\text{-}Abl}$ cells is due to the Bcr-Abl-induced protein degradation, because the expression of wild type Abi2 can be rescued by the Bcr-Abl kinase inhibitor imatinib (Fig. 2C, lane 3) as well as by a proteasome inhibitor MG132 (Fig. 2D).

To determine if the Y213 is required for Abi2 ubiquitination, the $p185^{Bcr\text{-}Abl}$ cells transfected with the constructs expressing either HA-tagged wild type Abi2 or Abi2 Y213F were treated with proteasome inhibitor MG-132 and the cell lysates are subjected to immunoprecipitation (IP) with anti-HA antibody. The immunoprecipitates were then analyzed by western blot using anti-ubiquitin antibody. As shown in Fig. 2D, wild type, but not mutant, Abi2 is ubiquitinated, Thus, our data indicates that Y213 in Abi proteins is a major phosphorylation site of Bcr-Abl and is essential for Bcr-Abl-induced Abi protein ubiquitination and degradation.

Bcr-Abl-induced Abi2 degradation requires Cbl E3 ubiquitin ligases

Tanos *et al.* has reported that the Abi1 forms a complex with Cbl [20]. The sequence analysis of Abi proteins reveals a peptide flanking Y213 that conforms consensus sequences that bind to Cbl tyrosine kinase binding (Cbl-TKB) motif [27] (Fig. 3 A). These findings raise the question whether the Cbl family E3 ubiquitin ligase may be involved in Bcr-Abl-induced Abi degradation. To address this question, we knocked out c-Cbl, Cbl-B, and both c-Cbl and Cbl-B genes in $p185^{Bcr\text{-}Abl}$ cells by CRISPR/Cas9-mediated gene editing. As shown in Fig. 3B, Ba/F3 and $p185^{Bcr\text{-}Abl}$ cells transfected with control plasmid (p185 ctrl) express both c-Cbl and Cbl-B (Fig. 3B, lanes 1 and 2). Consistent with the previous results, Abi2 is expressed in Ba/F3 cells but the expression is lost in $p185^{Bcr\text{-}Abl}$ control cells due to Bcr-Abl-induced degradation (Fig. 3B, compare lane 1 with lane 2). Depletion of either c-Cbl (Cbl KO, Fig. 3B, lanes 5 and 6) or Cbl-B (Cbl-B KO, Fig. 3B, lanes 7 and

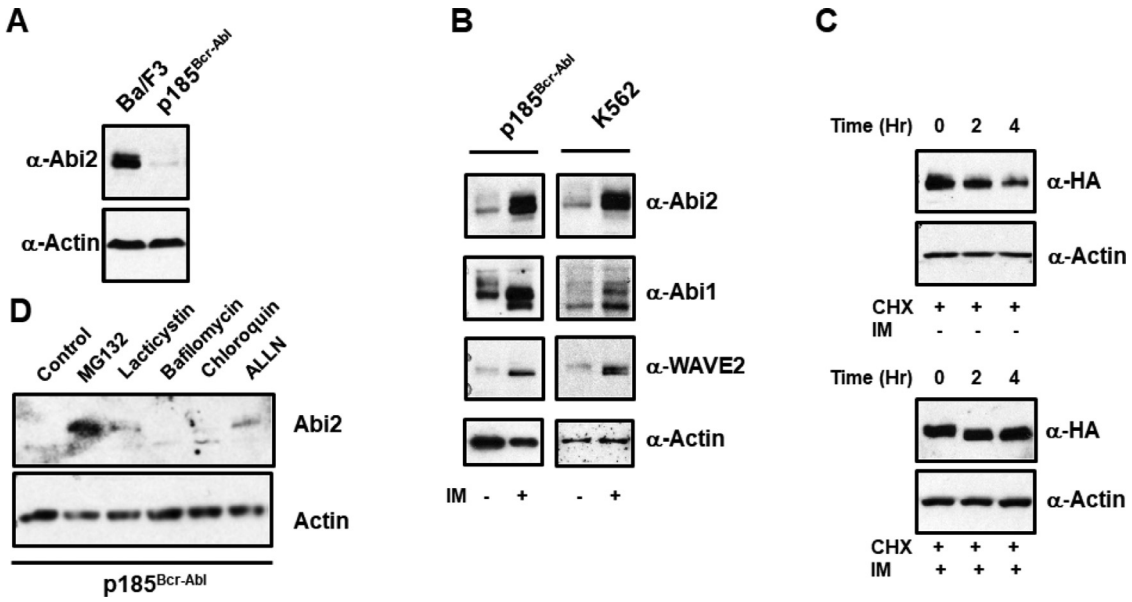

Fig. 1. Abl tyrosine kinases dependent degradation of Abi Proteins in Bcr-Abl-positive leukemic cells. A. Expression of $p185^{Bcr\text{-}Abl}$ in Ba/F3 cells induces down regulation of Abi2. Total lysates from 1×10^6 Ba/F3 and Ba/F3 expressing $p185^{Bcr\text{-}Abl}$ cells were analyzed by western blot using indicated antibodies. B. Abl tyrosine kinase inhibitor imatinib (IM) reverts Bcr-Abl-induced down-regulation of Abi and WAVE2 proteins. Ba/F3 $p185^{Bcr\text{-}Abl}$ and K562 cells were treated with or without 5 μM Abl kinase inhibitor imatinib (IM) for 8 h. Total lysates of 1×10^6 cells were analyzed by western blot using indicated antibodies. C. Imatinib (IM) treatment increases Abi1 protein level in $p185^{Bcr\text{-}Abl}$-positive leukemic cells. The $p185^{Bcr\text{-}Abl}$ cells expressing HA-tagged Abi1 were treated with or without 5 μM Abl kinase inhibitor imatinib (IM), as indicated, at the presence of 50 μM cycloheximide (CHX) for indicated hours. Total lysates of 1×10^6 cells were analyzed by western blot using indicated antibodies. D. Proteasome inhibitors revert Bcr-Abl-induced Abi2 down regulation. The $p185^{Bcr\text{-}Abl}$ cells were treated with proteasome inhibitors MG132 (20 μM) and lactacystin (10 μM), lysosome inhibitors bafilomycin A1 (1 μM) and chloroquine (100> μM), and calpain inhibitor ALLN (25 μM), as indicated, for 5 >h. Total lysates from 1×10^6 cells were subjected to western blot analysis.

8) alone in these cells did not rescue the Abi2 expression. However, when both c-Cbl and Cbl-B were knocked-out (Cbl/Cbl-B KO, Fig. 3B, lanes 3 and 4), the expression of Abi2 was fully rescued. Moreover, double knockout of c-Cbl and Cbl-B also increases the expression of Abi1 and WAVE2 in $p185^{Bcr\text{-}Abl}$-transformed Ba/F3 cells (Fig. 3C). Together, these studies show that Cbl family of E3 ubiquitin ligase is required for Bcr-Abl-induced down-regulation of Abi1, Abi2, and WAVE2.

Abi1 depletion in Bcr-Abl positive leukemic cells leads to down regulation of WAVE2 as well as inhibition of PI3K/Akt and Src family kinase Lyn pathways

Abi family of proteins is a component of WRC [12,13]. Studies from several laboratories have shown that depletion of either Abi, Hem/Sra, or WAVE will lead to down-regulation of WRC [18]. To determine the effect of down-regulation of Abi proteins on the WRC signaling, we knocked out Abi1 expression in $p185^{Bcr\text{-}Abl}$-transformed Ba/F3 cells and K562 cells by CRISPR/Cas9 gene editing. The depletion of Abi1 resulted in a remarkable down-regulation of WAVE2 in $p185^{Bcr\text{-}Abl}$-transformed Ba/F3 cells, as we reported previously [22], and in Bcr-Abl-positive K562 human leukemic cells (Fig. 4 A).

Cbl E3 ubiquitin ligase regulates Src family tyrosine kinases activity through its binding to phosphorylated tyrosine in Cbl-TKB binding motif of Src family proteins and thereby promoting ubiquitin-dependent degradation of activated Src kinases [28–31]. Because Abi proteins also contain a Cbl-PKB binding motif similar to that found in Src family kinases (Fig. 3A), it is possible that Y213-phosphorylated Abi proteins may compete with activated Src kinases to bind to Cbl. This may protect activated Src from being degraded by Cbl-mediated proteolysis, a mechanism found for Sprouty 2 protein [32,33]. To test the effect of Abi depletion on the Src family kinases signaling, we examined the activation of Src family kinases in Abi1 depleted $p185^{Bcr\text{-}Abl}$ cells and K562 cells where the complete knockout of Abi1 and remarkable down regulation of Abi2 allow us to test if Abi proteins are involved in the Src signaling. As shown in Fig. 4B, control $p185^{Bcr\text{-}Abl}$ cells and K562 cells express Src family kinase Lyn and the activation of Lyn was detected in these cells using a specific antibody (pSrc Y416) that recognize activated Lyn (Fig. 4B, lanes 1 and 4). The activation of Lyn in the Abi1-depleted $p185^{Bcr\text{-}Abl}$ cells and K562 cells, however, is reduced as compared to that in $p185^{Bcr\text{-}Abl}$ control cells and K562 control cells (Fig. 4B, compare lanes 2 and 3 to lane 1 and lanes 5 and 6 to 4, respectively).

Fang *et al.* has reported that Cbl-B binds to the p85 subunit of phosphoinositide 3-kinase (PI3K) and thereby regulates the activity of PI3K [34]. Additionally, Abi1 has been shown to bind to the p85 subunit of PI3K as well [26,35]. To determine the effect of Abi depletion on the PI3K/Akt signaling, we examined the activation of Akt in Abi1 depleted $p185^{Bcr\text{-}Abl}$ cells and K562 cells. As shown in Fig. 4C, the depletion of Abi1 in K562 cells leads to a decrease of Akt activation. A similar result was also observed in Abi1 depleted $p185^{Bcr\text{-}Abl}$ cells [22].

Abi1 is involved in the EGF/EGFR signaling in metastatic breast cancer cells

In addition to Src family kinases, the EGFR also contains a Cbl-TKB binding motif (Fig. 3A). We have previously shown that the Abi1 knockdown in MDA-MB-231 triple negative human breast cancer cells impaired the Src-ID1 signaling, a pathway downstream of EGFR [36]. The finding that Abi1 contains a Cbl-TKB binding motif similar to that found in EGFR (Fig. 4A) led us to examine if the Abi 1 is involved in the regulation of EGFR in breast cancer cells. To this end, we first tested if Abi1 interacts with EGFR in 231Br cells, a brain-metastatic subline of MDA-MB-231 cells.

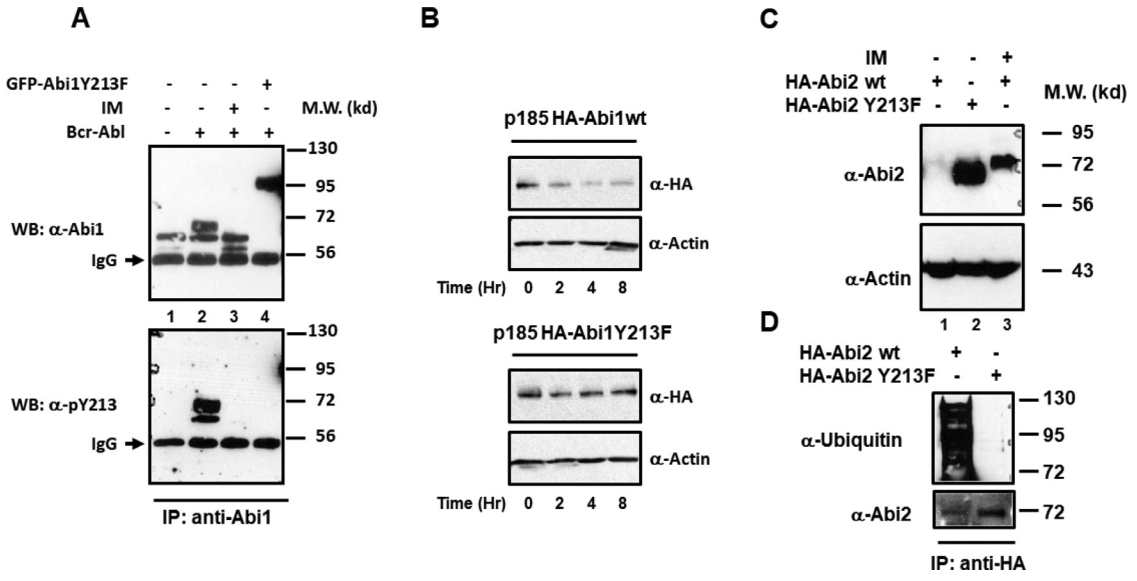

Fig. 2. The phosphorylation of tyrosine 213 is required for Bcr-Abl-induced degradation of Abi1 and Abi2. A. Phosphorylation of Abi1 tyrosine 213 (Y213) in $p185^{Bcr\text{-}Abl}$ cells. Ba/F3 cells and Ba/F3 cells expressing $p185^{Bcr\text{-}Abl}$ or $p185^{Bcr\text{-}Abl}$ plus green fluorescence protein (GFP)-tagged Abi1, as indicated, were treated with or without 5 μM imatinib (IM) for 8 h. Cell lysates were subjected to immunoprecipitation (IP) using anti-Abi1 antibody and the immunoprecipitates were analyzed by western blotting using an antibody raised against a peptide flanking phosphorylated tyrosine 213 (pY213). B. A mutation of Y213 to phenylalanine (Y213F) increases Abi1 protein stability in Bcr-Abl-positive leukemic cells. The $p185^{Bcr\text{-}Abl}$ cells expressing the HA-tagged wild type Abi1 (p185 HA-Abi1wt, upper panel) or Y213F mutant (p185 HA-Abi1Y213F, lower panel) were treated with 50 μM cycloheximide for indicated hours. Total lysates of 1×10^6 cells were analyzed by western blot using indicated antibodies. C. A mutation of Y213 to phenylalanine (Y213F) abolishes Bcr-Abl-induced degradation of Abi2. The $p185^{Bcr\text{-}Abl}$ cells stably expressing the HA-tagged wild type Abi2 (HA-Abi2 wt) or Y213F mutant (HA-Abi2Y213F) were treated with or without 5 μM imatinib (IM) for 8 h. Total lysates of 1×10^6 cells were analyzed by western blot using indicated antibodies. D. The Y213F mutation reduces Abi2 ubiquitination in $p185^{Bcr\text{-}Abl}$ cells. The $p185^{Bcr\text{-}Abl}$ cells stably expressing the HA-tagged wild type Abi2 (HA-Abi2 wt) or Y213F mutant (HA-Abi2Y213F) were treated with or without 50 μM MG132 for 5 h. Cell lysates were subjected to immunoprecipitation (IP) using anti-HA antibody and the immunoprecipitates were analyzed by western blotting using the indicated antibodies.

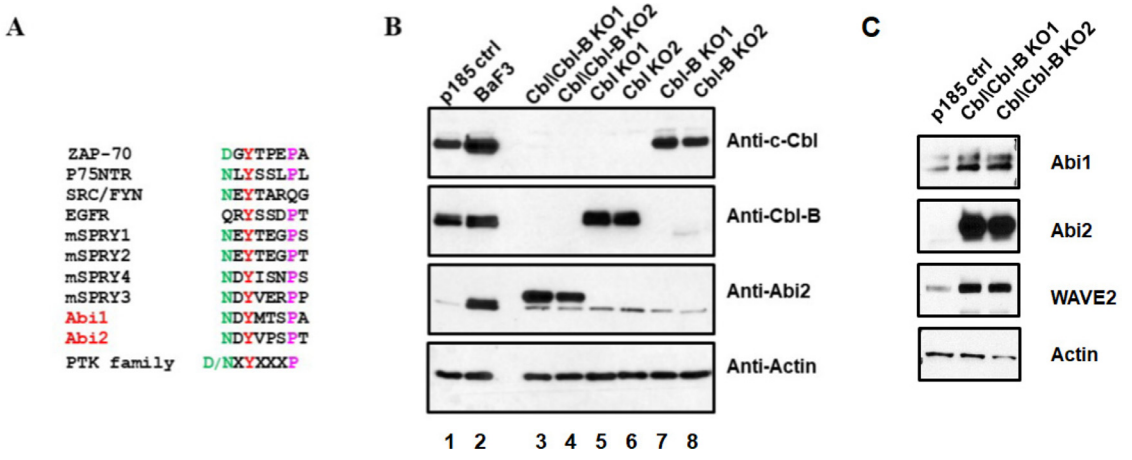

Fig. 3. Abi proteins contain a Cbl-TKB binding motif and Bcr-Abl induced downregulation of WRC requires Cbl E3 ubiquitin ligase. A. The alignment of amino acid sequences found in Abi1, Abi2, and various proteins that have been shown to bind to the Cbl-TKB domain. The consensus motif found in a cohort of protein tyrosine kinases (PTK) family is shown at the bottom. The highly conserved aspartic acid (D)/asparagine (N), tyrosine (Y), and proline (P) are in green, red, and purple, respectively, whereas the X represents any amino acid. B. Double knockout of c-Cbl and Cbl-B in $p185^{Bcr\text{-}Abl}$ cells rescues Abi2 from Bcr-Abl-induced degradation. Total lysates from 1×10^6 Ba/F3, $p185^{Bcr\text{-}Abl}$ control (p185 ctrl), and two independent lines of $p185^{Bcr\text{-}Abl}$ cells in which c-Cbl (Cbl KO1 and KO2), Cbl-B (Cbl-B KO1 and KO2), or both c-Cbl and Cbl-B (Cbl/Cbl-B KO1 and KO2) have been knocked out by CRISPR/Cas9-mediated gene editing were subjected to western blot analysis using the indicated antibodies. C. Double knockout of c-Cbl and Cbl-B in $p185^{Bcr\text{-}Abl}$ cells increases protein levels of Abi1, Abi2, and WAVE2. Total lysates from 1×10^6 $p185^{Bcr\text{-}Abl}$ control (p185 ctrl) and two independent lines of $p185^{Bcr\text{-}Abl}$ c-Cbl/Cbl-B double knockout cells (Cbl/Cbl-B KO1 and KO2) were subjected to western blot analysis using the indicated antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

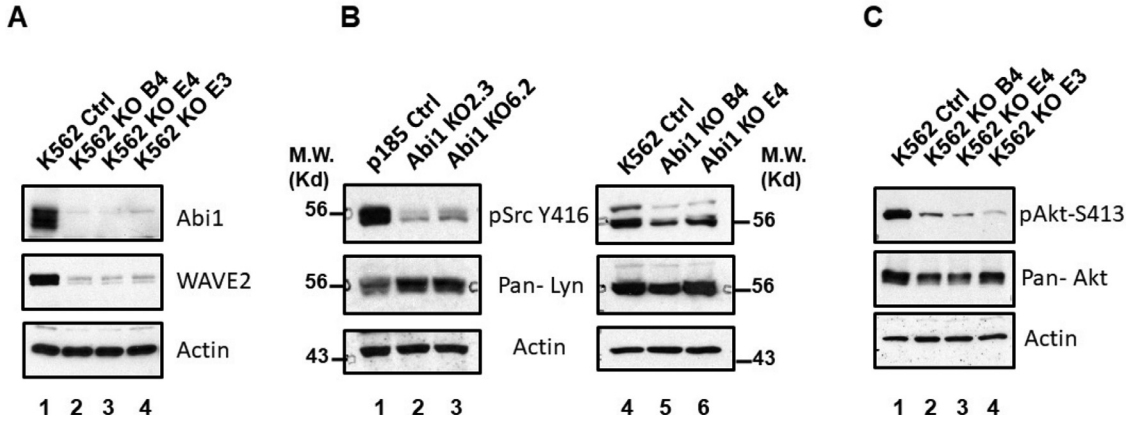

Fig. 4. Abi1 depletion downregulates WAVE2 and inhibits Akt and Lyn pathways in Bcr-Abl positive leukemic cells. A. Downregulation of WAVE2 in Abi1-depleted K562 cells. Total lysates from 1×10^6 control K562 (K562 ctrl) and three independent clones of Abi1-depleted K562 cells (K562 KO B4, E4, and E3) were analyzed by western blot using indicated antibodies. B. Depletion of Abi1 in $p185^{Bcr\text{-}Abl}$ cells (left panel) and K562 cells (right panel) reduces Lyn activation. Total lysates from 1×10^6 $p185^{Bcr\text{-}Abl}$ control cells (p185 ctrl), K562 control cells (K562 ctrl), two independent clones of Abi1-depleted $p185^{Bcr\text{-}Abl}$ cells (Abi1 KO2.3 and Abi1 KO6.2), and two independent clones of Abi1-depleted K562 (K562 KO B4 and KO E4) were analyzed by western blot using indicated antibodies. C. Abi1 knockout in K562 cells inhibits the activation of Akt pathway. Total lysates from 1×10^6 K562 control (K562 ctrl) and three clones of Abi1-depleted K562 cells (K562 KO B4, KO E4 and KO E3) were analyzed by western blot using indicated antibodies.

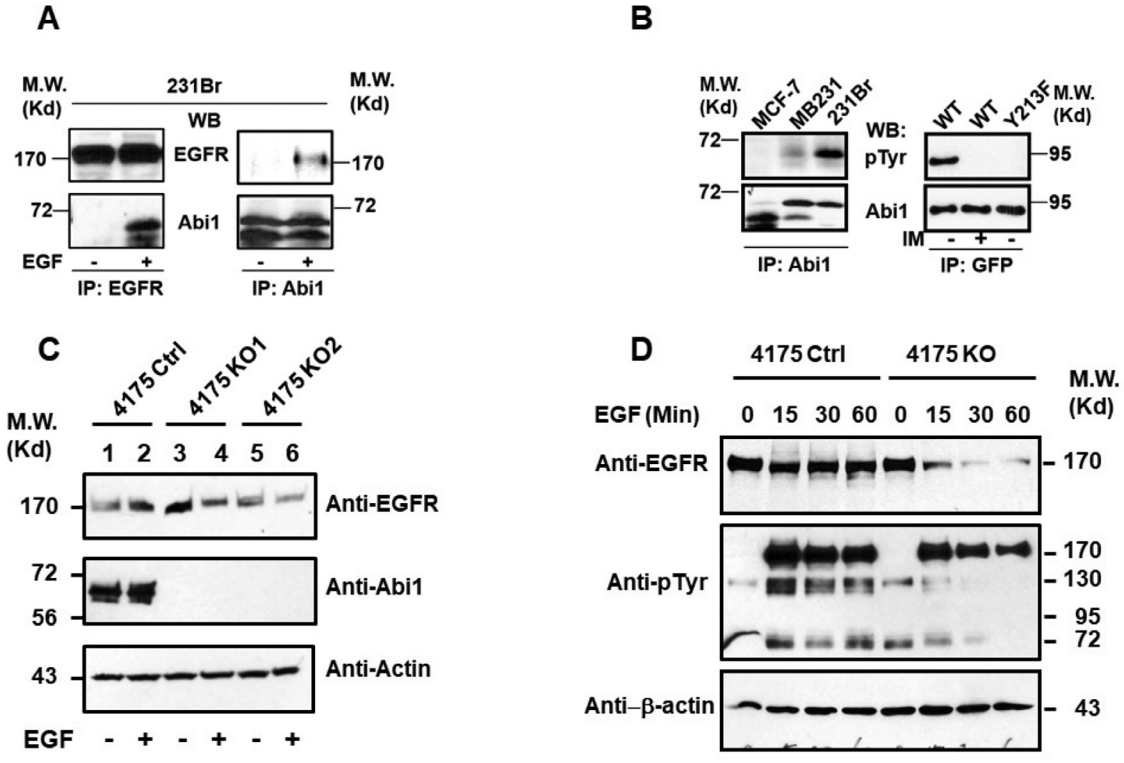

Fig. 5. Abi1 is involved in the regulation of the EGF/EGFR signaling in metastatic breast cancer cells. A. Abi1 binds to EGFR in metastatic MDA-MB-231 human breast cancer cells upon EGF stimulation. A brain metastatic subline of MDA-MB-231 cells (231Br) was stimulated with or without 50 ng/ml EGF for 30 min. The total lysates were subjected to immunoprecipitation (IP) followed by western blot (WB) analysis using antibodies as indicated. B. Abi1 is tyrosine-phosphorylated and the Y213 is a major site phosphorylated by Abl tyrosine kinases in metastatic breast cancer cells. Left panel: lysates of human breast cancer cell lines MCF-7 (MCF-7), MDA-MB-231 (MB231), and a brain metastatic subline of MDA-MB-231 (231Br) cells were subjected to immunoprecipitation (IP) followed by western blot (WB) analysis using indicated antibodies. Right panel: A lung metastatic subline of MDA-MB-231 cells, LM2-4175 was transduced with retroviruses expressing GFP-tagged wild type Abi1 (WT) or Abi1 Y213F mutant (Y213F). The cells were treated with or without 5 μM imatinib (IM), as indicated, for 8 h and total cell lysates were subjected to immunoprecipitation (IP) followed by western blot (WB) analysis using indicated antibodies. C and D. CRISPR/Cas9 mediated knockout of Abi1 in LM2-4175 breast cancer cells leads to EGFR down regulation. The control LM2-4175 cells (475 ctrl) and Abi1-depleted LM2-4175 cells (4175 KO) were stimulated with 50 ng/ml EGF for 60 min (C) or indicated time (D). Total cell lysates were analyzed by western blotting with indicated antibodies.

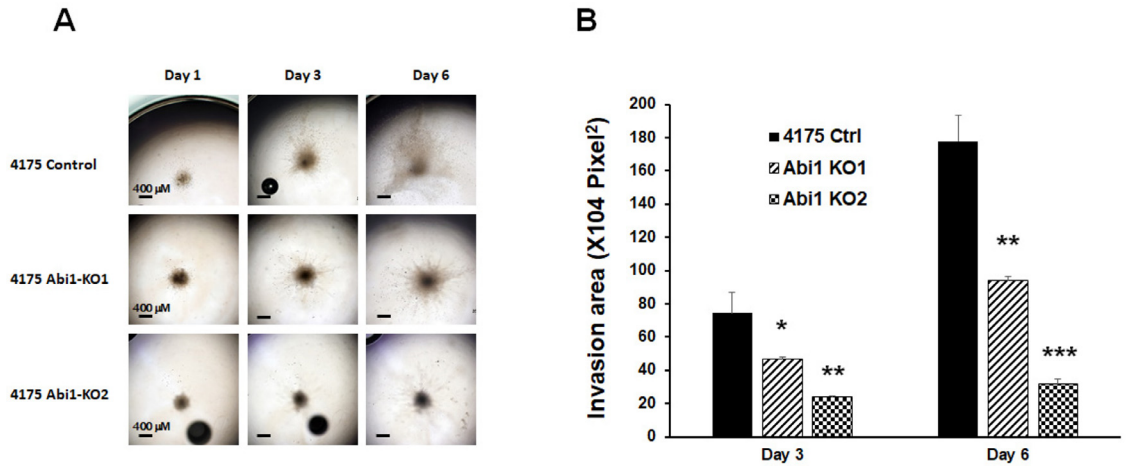

Fig. 6. Abi1 depletion impairs LM2-4175 breast cancer cells invasion in vitro. A. A 3D tumor spheroid invasion assay of control LM2-4175 cells (4175 Control) and 2 individual clones of LM2-4175 Abi1 knockout cells (4175 Abi1 KO1 and 4175 Abi1 KO2). The data is a representative of three independent experiments. B. Comparison of invasive area of control LM2-4175 cells (4175 Ctrl) and LM2-4175 Abi1 knockout cells (4175 Abi1 KO1 and 4175 Abi1 KO2) in a triplicate experiment. The invasion area was calculated using Adobe Photoshop software. Unpaired student t test was used to compare the knockout cells with the 4175 controls. * $P = 0.18$; ** $P < 0.05$; *** $P < 0.01$

Immunoprecipitation followed by western blotting analysis reveals that Abi1 is associated with EGFR upon EGF stimulation in these cells (Fig. 5A). Moreover, we found that, while the tyrosine phosphorylation of Abi1 is not detected in MCF-7 breast cancer cells which do not express EGFR, it is readily detected in metastatic MDA-MB-231 cells and a metastatic subline of MDA-MB-231, 231Br cells, both expressing high level of EGFR (Fig. 5B, left panel). The tyrosine phosphorylation of Abi1 in these metastatic breast cancer cells is mediated by Abl tyrosine kinase because the inhibition of Abl tyrosine kinases by imatinib abolished Abi1 tyrosine phosphorylation (Fig. 5B, right panel). Furthermore, we show that the Y213 in Abi1 is a major phosphorylation site because the mutation of Y213 to phenylalanine abolished the tyrosine phosphorylation of Abi1 in these cells (Fig. 5B, right panel).

To further define the role of Abi1 in regulating the EGF/EGFR signaling in human breast cancer cells, we knocked out *Abi1* gene in LM2-4175, a lung-metastatic subline of MDA-MB-231 human breast cancer cells (Fig. 5C). We then examined the level of EGFR in these cells with or without EGF stimulation. As shown in Fig. 5C and 5D, EGF induces a rapid activation of EGFR, as evidenced by the tyrosine phosphorylation of EGFR and its downstream targets, in both LM2-4175 control cells as well as LM2-4175 Abi1 KO cells (Fig. 5D, middle panel). However, in contrast to control LM2-4175 cells in which a prolonged activation and relatively more stable level of EGFR protein were observed, the LM2-4175 Abi1 knockout cells exhibited rapid downregulation of EGFR protein level and protein tyrosine phosphorylation after prolonged EGF stimulation (Fig. 5D).

Abi1 deficiency in breast cancer cells inhibits cell invasion in vitro and metastasis in vivo

To determine the role of Abi1 in breast cancer cell invasion and metastasis, we first examined the invasion of LM2-4175 control cells in a 3D spheroid assay and compared it to that of Abi1 depleted LM2-4175 cells. As shown in Fig. 6 A, LM2-4175 Cas9 control cells (4175 control) outgrew from the spheroid and invaded into surrounding matrix. In contrast, the outgrowth and invasion into surrounding matrix were reduced by 47% (S.D. +/-1.4%, $P < 0.05$) and 82% (S.D. +/-1.6%, $P < 0.01$) at day 6 in two independent Abi1-depeleted LM2-4175 clonal lines 4175 Abi1 KO1 and 4175 Abi1 KO2, respectively (Fig. 6A and 6B).

Next, we examined whether the depletion of Abi1 in LM2-4175 breast cancer cells affects their ability to metastasize in vivo. To this end, we injected the LM2-4175 Cas9 control (4175 ctrl) cells or two independent LM2-4175 Abi1 knockout (4175 KO1 and 4175 KO2) cell lines into the left cardiac ventricle of immune compromised Nu/Nu mice and monitored the cancer cell metastasis and disease development in these mice. The LM2-4175 cells are a subline of MDA-MB-231 cells selected by their ability to preferentially metastasize to lung [37]. As reported previously [37], the massive cancer cell metastasis to lung was observed in four out of five Nu/Nu mice injected with LM2-4175 control cells (Fig. 7 A-D, and Table 1). Metastases to the brain were also observed in two mice injected with LM2-4175 control cells (Fig. 7D and Table 1). These mice died or became moribund with a mean survival time of 39 days while the mice receiving saline remained healthy for over 65 days (Fig. 7E and Table 1). In contrast to the mice injected with LM2-4175 control cells, the mice injected with either LM2-4175 Abi1 KO1 or LM2-4175 Abi1 KO2 cells survived up to 65 days with neither signs of disease nor visible cancer cell metastasis (Fig. 7A-E and Table 1). The survival time was significantly different (Logrank test $P < 0.05$). Together, our in vitro and in vivo data support a role of Abi1 in breast cancer cell invasion and metastasis.

Discussion

In this study we investigated how the activated Abl tyrosine kinases regulate Abi protein stability and how the down-regulation of Abi proteins may affect the signaling mediated by WRC as well as receptor and non-receptor tyrosine kinases. Using Bcr-Abl-positive leukemic cell lines as a model we found that Abl-induced tyrosine phosphorylation is required for regulating Abi1 and Abi2 protein stability. We identified a motif in Abi1 and Abi2 that conforms to the consensus sequences found in many protein tyrosine kinases that bind to Cbl-TKB domain. We show that the Abl-induced phosphorylation of tyrosine 213 in this motif is essential for down regulation of Abi and WAVE proteins, the key components of WRC. Mutation of Y213 to phenylalanine in this motif blocked Abi1 and Abi2 degradation in $p185^{Bcr\text{-}Abl}$-positive Ba/F3 cells and double knockout of c-Cbl and Cbl-B in these cells rescued Abi and WAVE proteins from being degraded. Thus, our study shows that Abl tyrosine kinases link the

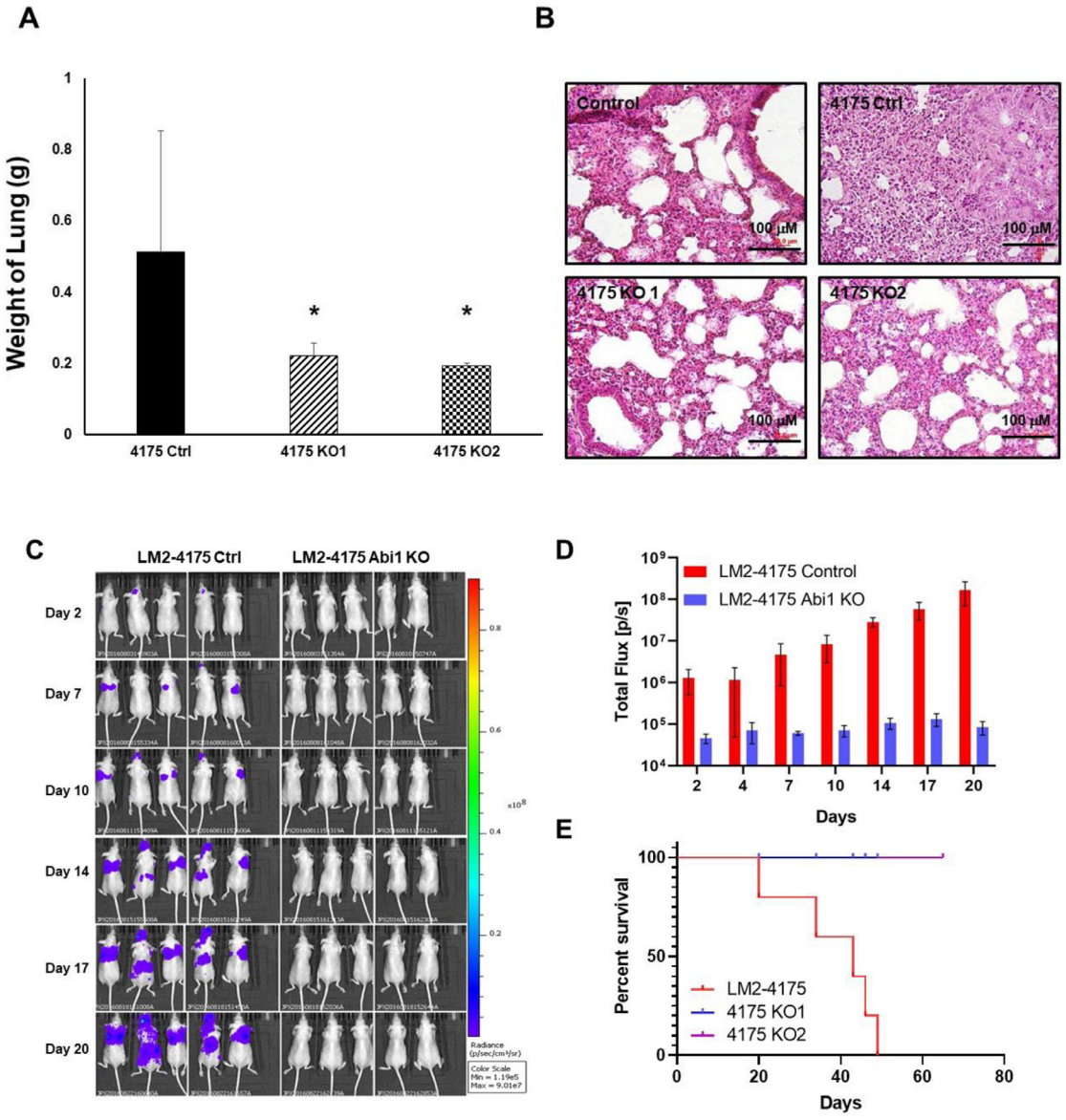

Fig. 7. Abi1 knockout impedes LM2-4175 breast cancer cells metastasis to lung in vivo. A. Lung weight of the mice injected intracardiacally with control LM2-4175 cells (4175 Ctrl, n = 5) and 2 individual clones of LM2-4175 Abi1 knockout cells (4175 KO1, n = 5; and 4175 KO2, n = 5; $^{*}P<0.05$ compared to 4175 Ctrl). B. H&E staining of the representative lungs from the mouse injected with saline (control), LM2-4175 control cells (4175 Ctrl), and two clones of LM2-4175 Abi1 knockout cells (4175 KO1 and 4175 KO2). C. Bioluminescence imaging of mice at 2, 7, 10, 14, 17, and 20 days after intracardiac injection of LM2-4175 (LM2-4175 Ctrl) or LM2-4175 Abi1 knockout (LM2-4175 Abi1 KO) cells. Scale bar denotes radiance in photons per second per square centimeter per steradian (p/sec/cm^2/sr). D. The quantitative bioluminescence imaging as the value of total flux (photons/sec) for mice injected with LM2-4175 control (red) and Abi1 knockout (4175 Abi1 KO, blue) cells. E. Survival curve of the mice injected with LM2-4175 cells (red, n = 5) and two individual clones of LM2-4175 Abi1 knockout cells, 4175 KO1 and 4175 KO2 (blue and purple, respectively, n = 5 for each group). Log-rank test $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

WRC to the Cbl E3 ubiquitin ligase-dependent proteolysis pathway by phosphorylating tyrosine 213 in a Cbl-TKB binding motif.

Wong *et al.* [33] and Rubin *et al.* [32] have reported that sprouty 2, a signaling molecule which also contains a Cbl-TKB binding motif, may function as a positive regulator of receptor tyrosine kinases by competitively binding to Cbl-TKB domain and thereby sequestering the active Cbl molecules and impeding the Cbl-mediated ubiquitination and degradation of the receptor tyrosine kinases. Given that Cbl-TKB binding motif has been found in a variety of receptor and non-receptor tyrosine kinases including EGFR, and Src family kinases, the finding that Abi1 and Abi2 also contain such a motif raises the question as to whether the Abi1 and/or Abi2 interplay with these signaling pathways during cancer development. To address this question we knocked out Abi1 in Bcr-Abl-positive leukemic cells as well as in triple negative breast cancer cells. We found that knockout of Abi1 in leukemic cells and breast cancer cells not only down-regulated WAVE2 protein but also reduced the activation of Src family kinases and the Akt pathway in p185$^{Bcr\text{-}Abl}$ positive leukemic cells. Moreover, the depletion of Abi1 in MDA-MB-231 human triple negative breast cancer cells downregulated EGFR protein level by enhancing EGF-stimulated EGFR degradation. These findings are consistent with a role of

Table 1

Summary of the disease development in mice injected with LM2-4175 control cells and the LM2-4175 Abi1 KO cells.

Mouse	Latency[a](days)	Lung weight (g)	Metastasis[e]
Saline Ctrl			
A1	64[b]	0.26	None
A2	64[b]	0.19	None
LM2-4175 Ctrl			
A1	46[c]	0.94	Lung
A2	23[c]	0.17	Brain
A3	49[d]	0.68	Lung
A4	34[c]	0.16	Lung, Brain
A5	43[c]	0.62	Lung
LM2-4175-Abi1 KO1			
A1	65[b]	0.22	None
A2	65[b]	0.28	None
A3	65[b]	0.21	None
A4	65[b]	0.22	None
A5	65[b]	0.18	None
LM2-4175-Abi1 KO2			
B1	58[b]	0.19	None
B2	58[b]	0.18	None
B3	65[b]	0.20	None
B4	65[b]	0.20	None
B5	65[b]	0.20	None

[a] Latency is defined as the time post-injection that mice died or become moribund.
[b] The day euthanized without any sign of disease.
[c] The mice found moribund at the day of pathology analysis.
[d] The mouse found dead at the day of pathology analysis.
[e] Cancer cell metastasis is determined by bioluminescence imaging analysis and gross pathology analysis.

Abi proteins in protecting receptor and non-receptor tyrosine kinases from Cbl-mediated proteolysis. Importantly, Abi1 knockout in LM2-4175, a lung metastatic subline of MDA-MB-231 cells, inhibited the cell invasion in a 3D spheroid invasion assay and the cancer cell metastasis to lung in nude mice. Taken together, our studies may help to explain how the Abl-Abi signaling contributes to the regulation of receptor and non-receptor tyrosine kinases pathways and cancer cell metastasis.

Actin cytoskeleton remodeling is a dynamic cellular process that is spatiotemporally regulated in normal cells but its deregulation is often observed in cancer cells. Highly dynamic nature of this process requires rapid switch on and off of the regulatory machine. Although it is well known that WRC is a key regulator of the actin cytoskeleton remodeling, how it regulates this highly dynamic process is not completely understood. Studies by Eden et al [12] suggest that WRC is inactive as a complex and dissociation of WAVE protein from other WRC components releases it from an intra-complex inhibitory interaction and thereby activates its actin nucleation promoting activity. This WRC activation mechanism is further supported by subsequent molecular and structural studies [15,17]. Dissociation and activation of WRC may occur as a result of the interaction with active Rac proteins or as an event of Abi tyrosine phosphorylation [12,15,38]. While this Rac- and/or Abi tyrosine phosphorylation-dependent activation model explains how WRC is switched on, it remains elusive with regard to the mechanism by which WRC is rapidly switched off following its activation, which is equally important for the actin cytoskeleton dynamics. Genetic studies have shown that while WRC is stable in cells as a complex, depletion of Abi proteins leads to degradation of WAVE proteins and *vice versa* [18]. Therefore, rapid degradation of Abi proteins may serve as a mechanism to switch off the WRC signaling. Thus, the finding that Abi tyrosine phosphorylation activates WRC activity, together with our finding that Abl tyrosine kinase-dependent Y213 phosphorylation of Abi1 and Abi2 promotes WRC degradation may provide a mechanism by which WRC is rapidly switched on and off.

Using a conditional knockout mouse model, Chorzalska et al show that bone marrow (BM)-specific loss of Abi1 results in abnormal hematopoietic cell development including anemia, premature exhaustion of BM hematopoietic stem cells, myeloproliferative neoplasm, and defects in B cell development [39]. Similar phenotypes were also observed in an earlier study by Park et al in which they knocked out Hem1, another component of WRC, in mouse [40]. They showed that depletion of Hem1 results in degradation of Abi1 and WAVE2. Remarkably, Hem1-deficient mice also exhibit anemia, lymphopenia, neutrophilia, and defects of lymphoid B and T cell development. More recently, Shao et al show that Hem1 and WRC are required for transition of fetal liver hematopoiesis to BM [41]. Although these studies highlight the importance of the Abi/WRC signaling in hematopoietic stem cell development, the mechanism involved is not clear. The findings that Abi proteins contain a Cbl-TKB binding motif and that Y213 phosphorylation by Abl kinases links the Abi/WRC pathway to the Cbl signaling raise a question as to whether the interplay of the Abi and Cbl signaling may play a role in hematopoietic stem cell development. In this regard it is notable that among the receptor tyrosine kinases that is regulated by Cbl, c-kit, a receptor tyrosine kinase essential for hematopoietic stem cell growth and differentiation, also contains a Cbl-TKB binding motif. Further studies are needed to determine if the Abi pathway is involved in the regulation of the c-kit signaling.

Although the Bcr-Abl transformation in Ba/F3 cells leads to rapid degradation of Abi2, we noticed that the down-regulation of Abi1 in these cells is moderate. It is likely that in addition to the Cbl-mediated proteolysis, other pathways yet to be identified may contribute to the regulation of Abi1 protein stability. Qi et al recently reported that PTEN-mediated dephosphorylation of serine 216 and tyrosine 213 promotes calpain-dependent proteolysis of Abi1 during embryo development [42]. It remains to be elucidated whether this pathway contributes to the regulation of Abi1 stability in cancer cells and leukemic cells.

Author contribution

PJ: Investigation, Data Curation, Formal Analysis, Methodology and Writing-review & editing; **ST, HH, TS, and AK**: Investigation and Methodology; **JD and XZ**: Formal Analysis and Writing-review & editing; **TN:** Funding acquisition and Writing-reviewing and editing; **XL**: Data Curation, Formal Analysis, Funding acquisition, Methodology, Supervision, and Writing-review & editing; **ZD:** Conceptualization, Data Curation, Formal Analysis, Funding acquisition, Investigation, Supervision, and Writing-original draft.

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