The N-terminal domain of the non-receptor tyrosine kinase ABL confers protein instability and suppresses tumorigenesis

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Chromosome translocation can lead to chimeric proteins that may become oncogenic drivers. A classic example is the fusion of the BCR activator of RhoGEF and GTPase and the ABL proto-oncogene nonreceptor tyrosine kinase, a result of a chromosome abnormality (Philadelphia chromosome) that causes leukemia. To unravel the mechanism underlying BCR-ABL–mediated tumorigenesis, here we compared the stability of ABL and the BCR-ABL fusion. Using protein degradation, cell proliferation, 5-ethynyl-2-deoxyuridine, and apoptosis assays, along with xenograft tumor analysis, we found that the N-terminal segment of ABL, which is lost in the BCR-ABL fusion, confers degradation capacity that is promoted by SMAD-specific E3 ubiquitin protein ligase 1. We further demonstrate that the N-terminal deletion renders ABL more stable and stimulates cell growth and tumorigenesis. The findings of our study suggest that altered protein stability may contribute to chromosome translocation-induced cancer development.

More than 500 chromosome translocation events have been tied to cancers, accounting for ~20% of malignant tumors (1, 2). Significant proportions of chronic myeloid leukemia and acute lymphocytic leukemia cases are tied to the Philadelphia chromosome translocation (3), a reciprocal exchange of DNA between chromosome 22 and chromosome 9, producing a chimeric protein, BCR-ABL, with tyrosine kinase activity (4–6). BCR-ABL expression in the blood-forming myeloid or lymphoid cells triggers leukemia (5, 7). As leukemia cells accumulate in the bone marrow, they can spill over into the blood and spread to other organs (5, 6).

The *BCR-ABL* gene is a fusion product of *BCR* (breakpoint cluster region) with *ABL* (Abelson proto-oncogene) caused by genomic rearrangement (6). The BCR-ABL chimera leads to an abnormally active kinase that elicits multiple downstream events that promote tumor growth and proliferation, including the RAS-RAF-MAPK pathway, the PI3K-Akt-BCL2 pathway, and the JAK-STAT pathway (8–10). BCR-ABL–triggered inappropriate activation of a signaling cascade has far-reaching effects on cell growth, proliferation, angiogenesis, and invasion, which are key to cancer development and progression (4, 9). Therefore, targeting BCR-ABL for therapeutic purposes has been an important focus for the pharmaceutical industry (6, 9).

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The BCR-ABL fusion derives from a combination of the removal of a small N-terminal ABL fragment (*i.e.* first 45 amino acids) and the addition of a part of the BCR protein. The tyrosine kinase activity of BCR-ABL, which is essential for its oncogenic potential, largely derives from ABL tyrosine kinase (6, 10). It is crucial to understand how the BCR-ABL fusion turns the ABL tyrosine kinase activity constitutively active (6, 10). Differential regulation of ABL and BCR-ABL may contribute to BCR-ABL–mediated tumorigenesis. Here, we evaluate the role of protein degradation in the regulation of ABL and BCR-ABL. ABL is rapidly degraded by the proteasome, which is promoted by the SMAD-specific E3 ubiquitin protein ligase 1 (Smurf1) ubiquitin ligase and is also dependent on the first 45 amino acids of ABL, which are missing in the BCR-ABL fusion. The N-terminal fragment of ABL bears a degradation signal that can render GFP protein unstable. We demonstrate that the first 45 amino acids of ABL are required for the binding to Smurf1 ubiquitin ligase and include lysine residues crucial for ABL turnover. Furthermore, the $ABL^{\triangle 45}$ mutant promotes cell growth and survival, suggesting that the removal of the ABL N-terminal segment may contribute to BCR-ABL–mediated cell signaling. Importantly, about half of the xenograft mice expressing $ABL^{\triangle 45}$ mutant developed tumors. Our study reveals a novel function of the N-terminal region of ABL and shed light on the mechanism underlying BCR-ABL–triggered oncogenic events.

Results

ABL turnover is faster than that of BCR-ABL and is mediated by the ubiquitin-proteasome system

The *BCR-ABL* oncogene is a product of chromosome translocation between chromosome 9 and chromosome 22. Depending on the translocation breakpoint in the *BCR* gene, different BCR-ABL chimeras are produced, with the most common form being p210 (210 kDa) (4–6, 9). To evaluate the protein stability of ABL and BCR-ABL, we transfected plasmids bearing FLAG-tagged ABL or the GST-tagged p210 BCR-ABL fusion into HEK-293 cells separately. After protein synthesis was turned off by treatment with cycloheximide, we monitored the degradation kinetics of ABL and BCR-ABL (Fig. 1*A*) and found that ABL was degraded much faster than the BCR-ABL fusion (Fig. 1, *A* and *C*). We also examined the degradation of endogenous ABL and p210 BCR-ABL in TF-1 and K562 cells,

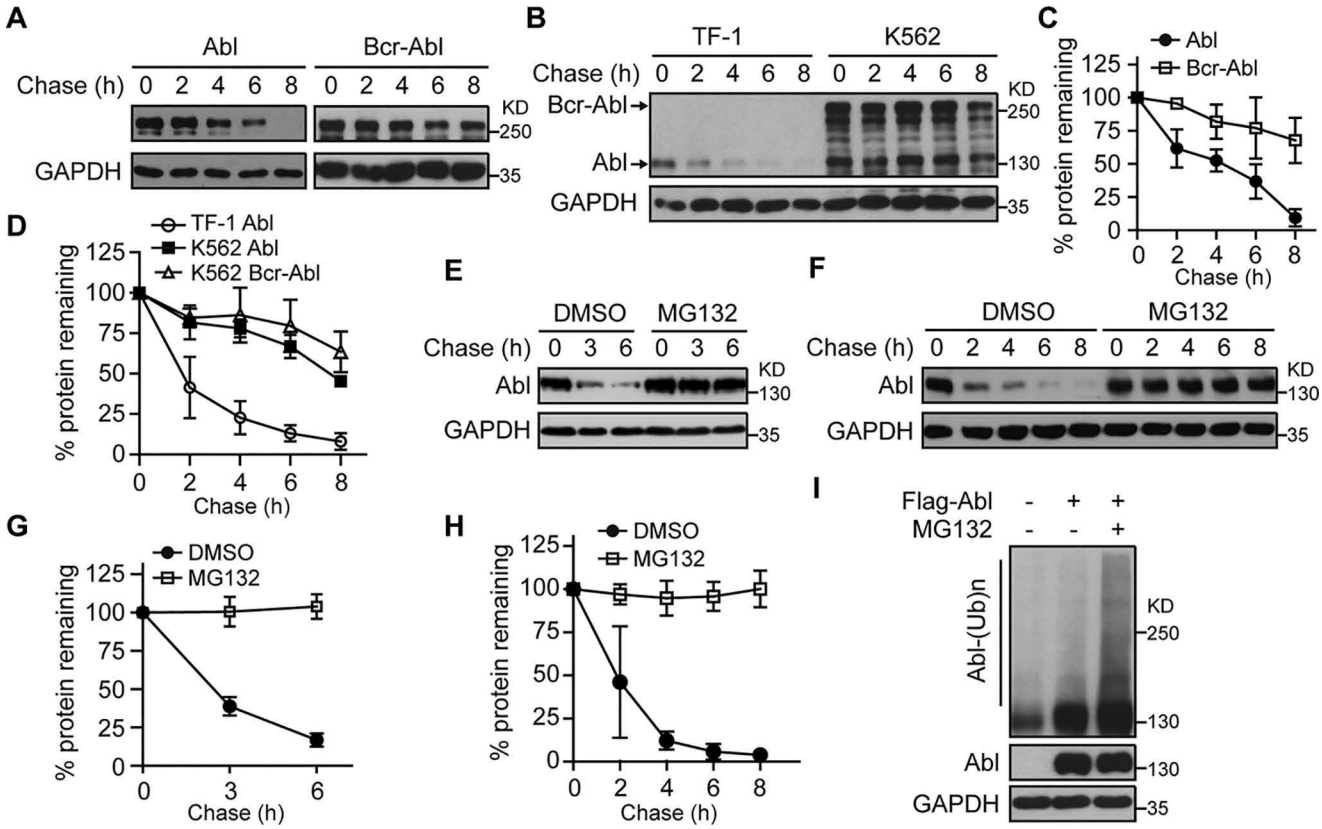

Figure 1. ABL is preferentially degraded by the ubiquitin proteasome system. *A*, degradation kinetics of ectopically expressed ABL and BCR-ABL fusion, analyzed by a protein expression shut-off assay. HEK-293 cells transfected with FLAG-ABL or GST-tagged BCR-ABL were treated with 100 μg/ml cycloheximide to shut off protein synthesis. Samples collected at various time points were then processed for immunoblotting with FLAG or GST antibody (*upper*). Similar amounts of protein extracts were used, as ascertained by blotting with anti-GAPDH (*lower*). All experiments were performed at least three times. *B*, protein stability of endogenous ABL and BCR-ABL in TF-1 or K562 cells, respectively. Degradation kinetics of endogenous ABL or BCR-ABL were determined as in *A* except that ABL antibody was used. *C* and *D*, quantification of the data in *A* and *B*, respectively. The results are derived from more than three experiments. *E* and *F*, ABL turnover is mediated by the proteasome. Degradation kinetics of ectopically expressed (*E*) or endogenous (*F*) ABL were analyzed in the absence or presence of the proteasome inhibitor MG132. *G* and *H*, quantification of the data in *E* and *F*, respectively. *I*, ABL is modified by ubiquitin. HEK-293 cells bearing FLAG-ABL with or without MG132 treatment were lysed and immunoprecipitated with FLAG antibody. Immunoprecipitates were then subjected to Western blotting with anti-ubiquitin to detect ubiquitylated ABL species (*upper*). The amounts of ABL and GAPDH control in protein extracts were also assessed by Western blotting (*lower*).

respectively. Consistent with the analysis of ectopically expressed ABL and BCR-ABL, endogenous ABL was degraded markedly faster than the BCR-ABL fusion (Fig. 1, *B* and *D*).

To assess whether ABL turnover requires the proteasome, we examined ABL degradation in the presence and absence of the proteasome inhibitor MG132. Both ectopically expressed and endogenous ABL proteins were dramatically stabilized upon MG132 treatment (Fig. 1, *E–H*), suggesting the involvement of the proteasome in ABL degradation. Proteasomal degradation of substrate proteins can be either ubiquitin dependent or ubiquitin independent. To evaluate whether ABL is modified by ubiquitin, HEK-293 cells were transfected with the plasmid bearing FLAG-tagged ABL or a control vector. Ubiquitylated ABL species were detected in cells expressing FLAG-ABL (Fig. 1*I*) and were enriched upon the addition of MG132 (Fig. 1*I*). These results suggest that ABL degradation is mediated by the ubiquitin-proteasome system.

The N-terminal region of ABL, missing in BCR-ABL fusions, is critical for ABL turnover

BCR-ABL is derived from the loss of the N-terminal 45 amino acids of ABL and the addition of a fragment of BCR (Fig. 2*A*) (6, 9). Given the distinct degradation rates of BCR-ABL and ABL (Fig. 1), we wondered whether the N-terminal segment is key to ABL turnover. To evaluate this conjecture, we assessed the stability of the ABL$^{\triangle 45}$ deletion mutant, lacking the first 45 amino acids. Interestingly, the N-terminal deletion rendered the ABL$^{\triangle 45}$ mutant much more stable than ABL (Fig. 2, *B* and *E*).

Is the N-terminal region of ABL enough to trigger substrate degradation? To address this issue, we fused the first 45 amino acids of ABL to the stable GFP. The N-terminal ABL served as a degradation signal, since the ABL^{45}-GFP fusion protein, unlike GFP, was degraded (Fig. 2, *C* and *F*). Furthermore, MG132 also stabilized the ABL^{45}-GFP chimera (Fig. 2, *D* and *G*). Combined, our results suggest that the N-terminal region of ABL bears a proteasomal degradation signal, which is lost not only in p210 but also in all other pathogenic BCR-ABL fusions.

The ubiquitin ligase Smurf1 promotes the ubiquitylation and degradation of ABL but not ABL$^{\triangle 45}$

The ubiquitin ligase Smurf1 was shown to promote ABL ubiquitylation, which triggers subsequent degradation (11). We then evaluated the relationship between Smurf1 and ABL

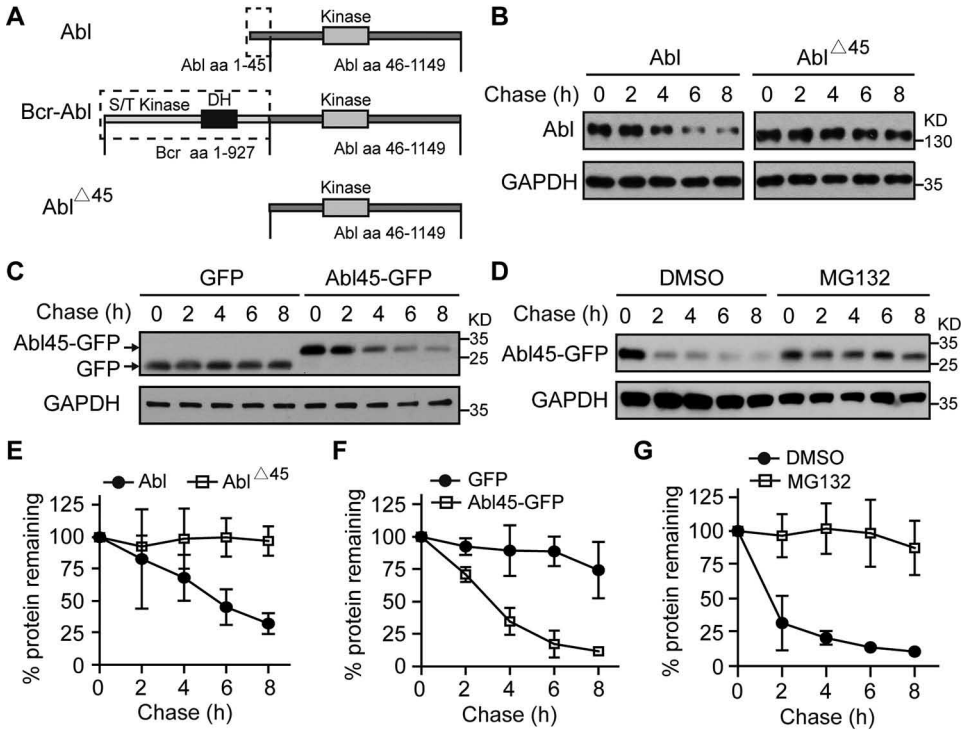

Figure 2. The degradation signal lies in the N-terminal domain of ABL. *A*, domain structures of ABL, BCR-ABL (p210), and ABL$^{\triangle 45}$. The first 45 amino acids of ABL are lost in the BCR-ABL fusion and deleted in ABL$^{\triangle 45}$. *B*, the N-terminal region is critical for ABL degradation. HEK-293 cells expressing FLAG-tagged ABL or ABL$^{\triangle 45}$ were processed as described in Fig. 1*A*, to determine the protein stability of ABL and ABL$^{\triangle 45}$. *C*, the N-terminal fragment of ABL confers protein instability. The degradation kinetics of GFP and ABL^{45}-GFP fusion were analyzed by the protein expression shut-off assay. *D*, the degradation induced by the N-terminal ABL fragment requires the proteasome. The stability of ABL^{45}-GFP was analyzed in the absence or presence of MG132. *E–G*, quantification of the data in *B–D*, respectively.

derivatives. Interestingly, Smurf1 overexpression caused ABL reduction, as expected (Fig. 3, *A* and *B*), but did not affect ABL$^{\triangle 45}$ or p210 BCR-ABL levels (Fig. 3, *C* and *D*), suggesting that Smurf1 action depends on the N-terminal ABL segment. Consistent with these observations, Smurf1 overexpression stimulates the ubiquitylation of ABL but not the ABL$^{\triangle 45}$ mutant (Fig. 3*E*). The data suggest that Smurf1-mediated ABL ubiquitylation and degradation require the N-terminal domain of ABL.

The N-terminal domain of ABL is required for the interaction with Smurf1 and contains lysine residues critical for ABL turnover

Substrates are selected by ubiquitin ligases for degradation through direct binding. Therefore, we examined whether the ubiquitin ligase Smurf1 interacted with ABL in coimmunoprecipitation assays (Fig. 3*F*). We found that Smurf1 immunoprecipitated ABL but not ABL$^{\triangle 45}$ (Fig. 3*F*), suggesting that the Smurf1-ABL association depends on the first 45 amino acids of ABL.

A ubiquitin ligase attaches ubiquitin onto lysine residue(s) of the substrate, triggering subsequent degradation. There are four lysine residues (*i.e.* K7, K24, K28, and K29) in the first 45 amino acids of ABL. We mutated these four lysines individually and collectively (Fig. 3, *G–I*). Smurf1 overexpression reduced the level of ABL but not that of the ABL mutant lacking all four lysines (Fig. 3*G*). Moreover, ABL turnover was impaired when all four lysines were mutated (Fig. 3, *H* and *I*). Combined, the data provide the molecular basis for the function of the N-terminal domain in ABL degradation.

Expression of the ABL$^{\triangle 45}$ mutant promotes cell growth and survival

The human erythroleukemic cell line TF-1 normally requires granulocyte/macrophage colony-stimulating factor (GM-CSF) for proliferation and survival (12, 13). To determine the functional consequences of ABL$^{\triangle 45}$ on cell growth and survival, we used lentivirus to generate stable TF-1 cells expressing BCR-ABL, ABL$^{\triangle 45}$, ABL, or negative control vector. In the presence of GM-CSF, all three ABL alleles enhanced cell proliferation, as measured by both direct cell counting and 5-ethynyl-2-deoxyuridine (EdU) assays, with ABL$^{\triangle 45}$ being more potent than ABL but weaker than p210 BCR-ABL (Fig. 4, *A–C*).

Without GM-CSF, TF-1 cells normally are quiescent and undergo apoptosis, but BCR-ABL–transduced TF1 cells can grow in the absence of growth factor (12, 13). Importantly, like BCR-ABL, ABL$^{\triangle 45}$, but not ABL, can drive growth factor-independent proliferation (Fig. 4*D*). We also examined whether ABL$^{\triangle 45}$ expression could inhibit GM-CSF deprivation-induced apoptosis. Interestingly, both ABL$^{\triangle 45}$ and BCR-ABL markedly reduced apoptosis (Fig. 4*E*), suggesting that ABL$^{\triangle 45}$ promotes cell growth and survival.

The ABL$^{\triangle 45}$ mutant promotes tumorigenesis

Next we evaluated whether ABL$^{\triangle 45}$ expression stimulated tumor development and growth *in vivo* (Fig. 5). Nude mice

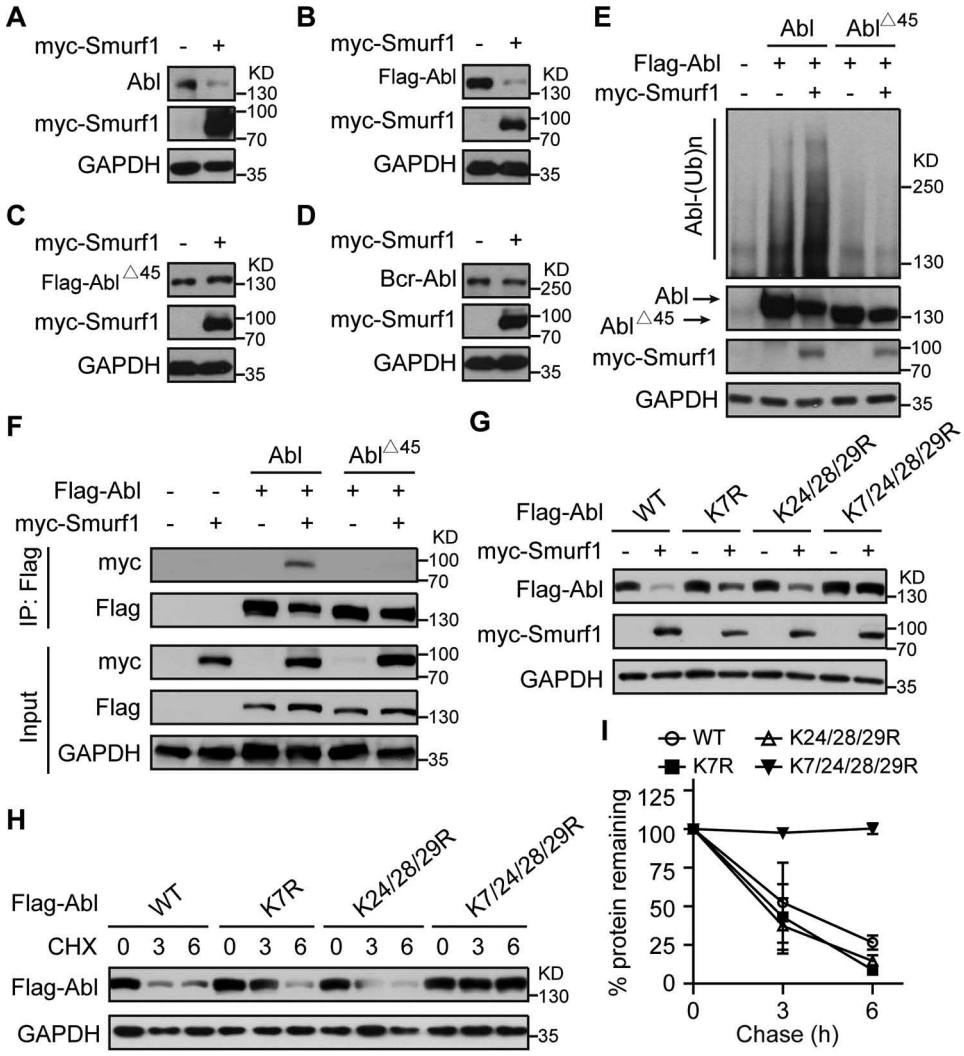

Figure 3. Ubiquitin ligase Smurf1 promotes ABL turnover via its N-terminal domain. *A–D*, Smurf1-stimulated ABL degradation requires its N-terminal region. Cells expressing endogenous ABL (*A*) or FLAG-tagged ABL (*B*) or ABL$^{\triangle 45}$ (*C*) or GST-tagged BCR-ABL (*D*) were transfected with a vector plasmid or the plasmid bearing Myc-tagged Smurf1. Levels of ABL derivatives, Myc-Smurf1, and GAPDH were assessed by Western blotting with various antibodies. *E*, Smurf1 promotes the ubiquitylation of ABL but not the ABL$^{\triangle 45}$ mutant. Ubiquitylation profiles of ABL and ABL$^{\triangle 45}$ with or without ectopic expression of Myc-Smurf1 were determined as described in Fig. 1*E*. *F*, the association between Smurf1 and ABL requires its N-terminal region. Cells expressing Myc-Smurf1 and/or FLAG-tagged ABL derivatives were harvested and lysed. Protein extracts were incubated with IgG beads coated with FLAG antibody. The immunoprecipitates were resolved by SDS-PAGE and processed for Western blotting using Myc or FLAG antibody as indicated. The inputs are shown in the *lower panels*. *G*, Smurf1-promoted ABL degradation requires lysine residues in the N-terminal region of ABL. Cells expressing FLAG-tagged WT ABL or the indicated lysine mutants were transfected with the plasmid with or without Myc-Smurf1. The effects on the expression of ABL derivatives were determined. *H*, four lysine residues in the N-terminal region are important for ABL degradation. The degradation kinetics of the indicated ABL derivatives were analyzed by the protein expression shut-off assay. *I*, quantification of the data in *H*.

were injected with TF-1 cells stably expressing BCR-ABL, ABL$^{\triangle 45}$, ABL, or vector control. The tumor take rates of BCR-ABL and vector were 100 and 0%, serving as positive and negative controls, respectively (Fig. 5). The tumor take rate for xenograft mice with ABL expression was 1 of 6, whereas the tumor take rate for mice with ABL$^{\triangle 45}$ expression was 3 of 6 (Fig. 5*A*). The effect of ABL derivatives on tumor growth was also evaluated by monitoring the tumor volume for mice bearing BCR-ABL, ABL$^{\triangle 45}$, ABL, or vector control (Fig. 5*B*). Tumor size was measured every other day. As expected, BCR-ABL promoted rapid tumor progression (Fig. 5*B*). ABL$^{\triangle 45}$ was much more potent than ABL in stimulating tumor growth (Fig. 5*B*). The results indicate that ABL$^{\triangle 45}$ expression promotes tumorigenesis *in vivo*.

Discussion

Intracellular protein homeostasis, which involves both protein synthesis and degradation, is essential for proper cell functioning (14, 15). Key cellular proteins, including Myc, Ras, p53, and cyclins, are selectively targeted to the proteasome for destruction (15). Misregulation of proteolysis can lead to diseases ranging from cancers to neurodegenerative disorders (15, 16). Here we explored the mechanism underlying ABL- and BCR-ABL–dependent oncogenic transformation. ABL contain the tyrosine kinase activity that is essential for the functioning of BCR-ABL in leukemia (6, 10). It is well established that chromosome translocation converts the ABL proto-oncogene to the BCR-ABL oncogene. Although the BCR addition is certainly important for BCR-ABL action in leukemia (2, 17), the

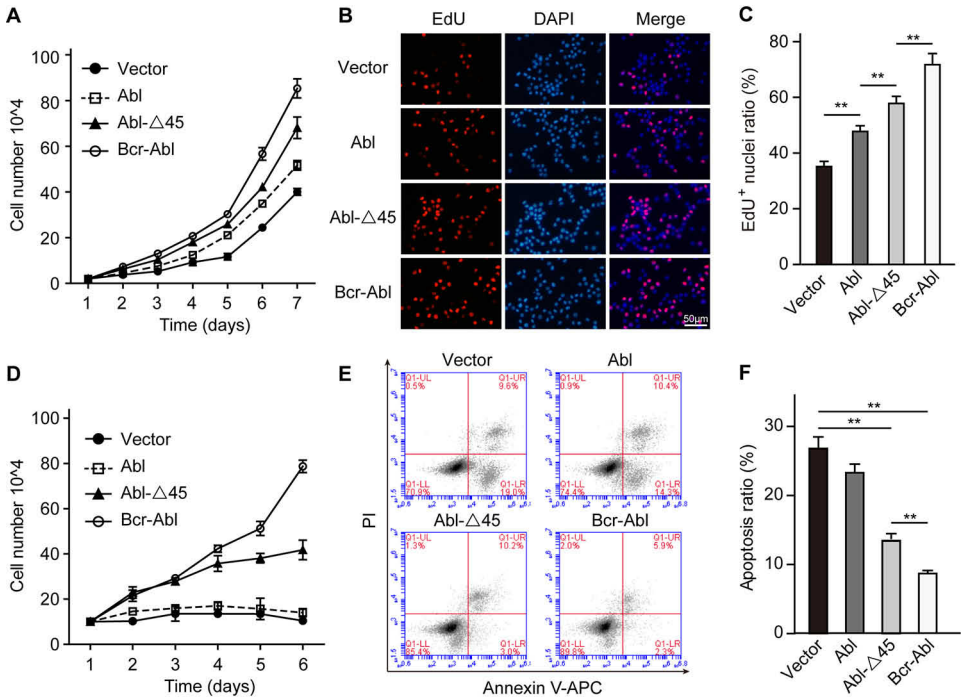

Figure 4. Effects of ABL derivatives on cell growth and survival. *A*, ABL derivatives promote TF-1 cell proliferation in the presence of GM-CSF. Cell proliferation was determined by hemocytometer measurements on the indicated days. *B*, effects of ABL derivatives on TF-1 cell growth in the presence of GM-CSF. The EdU assay was employed to measure DNA synthesis after cells were incubated for 24 h. EdU-positive cells were indicated by *red* fluorescence. 4[prime],6-Diamidino-2-phenylindole (*DAPI*) stains for nuclei (*blue*). *Bar*, 50 μM. *C*, quantification of the data in *B*. The percentage of EdU-positive nuclei, relative to the total number of nuclei, is shown. *p* values determined by student's *t* test are indicated, **, $p < 0.01$. *D*, BCR-ABL and ABL$^{△45}$, but not ABL, promote TF-1 cell proliferation in the absence of GM-CSF. Cell proliferation was performed as described in *A* except that cells were cultured in GM-CSF-free medium. *E*, effects of ABL derivatives on TF-1 cell death in the absence of GM-CSF. TF-1 cells expressing ABL derivatives were cultured in GM-CSF-free medium for 48 h and then stained with annexin V and propidium iodide to determine the apoptosis rate. *F*, quantification of the data in *E*.

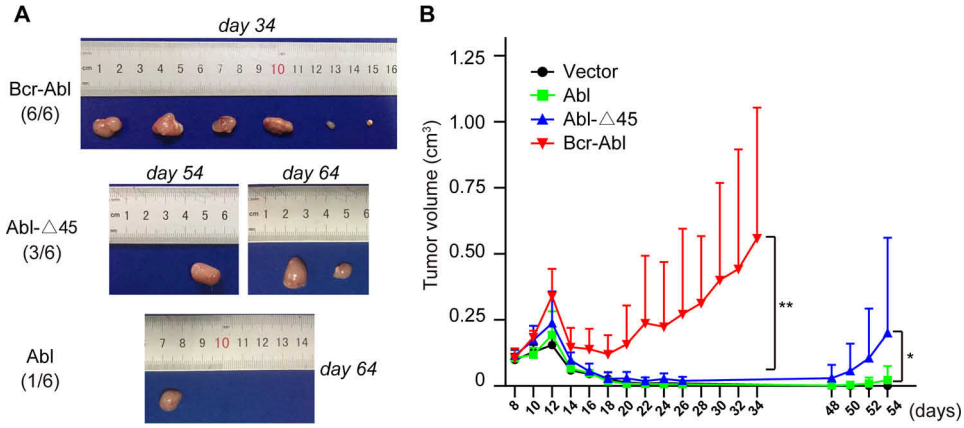

Figure 5. Tumor formation in mouse xenografts expressing ABL derivatives. *A*, tumors dissected from xenograft mice. TF-1 cells expressing ABL derivatives indicated were subcutaneously implanted into nude mice ($n = 6$ for each group). Tumor size was monitored by Vernier caliper. Different termination times were indicated, to prevent the tumors from growing too large. *B*, tumor growth curve in xenograft mice expressing ABL derivatives. Beginning at 7 days postinjection, tumor size was calculated every other day. BCR-ABL-expressing mice were sacrificed earlier because tumors grew quickly.

pathogenic contribution of the loss of the N-terminal ABL segment in cancer remains poorly understood and underestimated (6). Our study reveals an important role for the first 45 amino acids of ABL in keeping ABL levels under control via proteasome-mediated degradation, which in turn suppresses its transforming potency and tumor growth.

Three genetic mechanisms commonly employed to turn a proto-oncogene into an oncogene are gene amplification, mutation, and translocation (1). These genetic perturbations lead to either enhanced protein expression or altered protein structure and/or function. Our results indicate that the loss of the N-terminal ABL domain caused by chromosome translocation impedes ABL degradation (Figs. 1 and 2) and promotes the oncogenic potential of ABL (Figs. 4 and 5), which is further enhanced by the BCR addition (2, 10), highlighting the underappreciated role of translocation-induced protein stability in tumorigenesis. In several types of leukemia, ABL is found to be fused to other proteins, including NUP214, SNX2, FOXP1, and ETV6 (6,7,10). Although the breakpoints of these ABL fusions vary, they all lose the first 45 amino acids of ABL, which may be

a key contributor to leukemia development (6, 7). Hundreds of chromosome translocation events have been identified, accounting for ~20% of malignant tumors (1). It is likely that altered protein stability due to chromosome translocation could be a driving pathogenic factor in at least some of these cases.

As a key regulator of diverse cellular events, ABL activity is tightly controlled through multiple mechanisms, including transcription regulation, phosphorylation and dephosphorylation, interactions with positive facilitators (*e.g.* RIN1) and negative inhibitors (*e.g.* AAP1, Abi1), and protein degradation (6, 7, 10, 18). We have uncovered an important *cis*-element (*i.e.* the N-terminal region) and *trans*-factor (*i.e.* Smurf1 E3) critical for ABL turnover (Figs. 2 and 3) (11). Chromosome rearrangement upsets the delicate balance of ABL regulation, triggering leukemia (4, 5). Our results provide a novel biochemical basis for BCR-ABL–dependent tumorigenesis. Independent of the BCR-ABL translocation, elevated ABL activity has been linked to brain, colon, kidney, lung, and prostate cancers (6, 7, 19), which can explain the low incidence of tumor induction in the mice overexpressing ABL (Fig. 5). ABL$^{\triangle 45}$ expression led to ~50% of xenograft mice developing tumors, underscoring the functional significance of the N-terminal region of ABL.

Modulation of ABL and/or BCR-ABL stability may provide an effective strategy against cancers related to aberrant ABL activity. Targeting BCR-ABL for therapeutic purposes has been an important focus for the pharmaceutical industry (4, 6, 10). As a potent inhibitor of BCR-ABL tyrosine kinase activity, Gleevec (*i.e.* imatinib) is known for its phenomenal effect in early clinical trials, with its ability to halt the signaling cascade for cell growth, tumor proliferation, and migration (4, 10). Like many targeted therapies, however, the inhibition requires persistent binding between imatinib and BCR-ABL, and cells develop alternative route(s) to resume cell growth, leading to imatinib resistance and limiting its long-term efficacy. In this regard, the recent development of bivalent small molecules that rewire the ubiquitin pathway to trigger proteasome-mediated destruction of ABL and BCR-ABL may bring effective means to the battle against ABL- and BCR-ABL-related maladies (20–23).

Experimental Procedures

Cell culture

The K562 cell line (ATCC, Manassas, VA) was cultured in RPMI 1640 medium with 10% FBS (GE Healthcare, Chicago, IL). The TF-1 cell line was cultured in RPMI 1640 medium with 10% FBS and 2 ng/ml recombinant human GM-CSF. HEK-293 cells were cultured in DMEM with 10% FBS.

Plasmids, antibodies, and other reagents

The plasmids for pcDNA3-ABL-His$_6$-FLAG, pLEF-BCR-ABL (p210), and pRK-Myc-Smurf1 were obtained from Addgene (Cambridge, MA). The lentiviral plasmids for pEZ-Lv242 vector, pLV-ABL1, and pLV-BCR-ABL (p210) were purchased from GeneCopoeia (Rockville, MD). The first 45 amino acids of ABL were subcloned in-frame into pEGFP-N2, linking it to the N terminus of GFP. The ABL mutant lacking the first 45 amino acids was made with the Q5 site-directed mutagenesis kit (New England Biolabs, Ipswich, MA).

Antibodies against FLAG, Myc, and GAPDH were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against GST and c-ABL were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against GFP and HA were purchased from Abcam (Cambridge, MA) and BioLegend (San Diego, CA), respectively. Anti-ubiquitin antibody was purchased from Covance (Princeton, NJ). MG-132 is a proteasome inhibitor obtained from Calbiochem (Gibbstown, NJ). Cycloheximide and GM-CSF were purchased from Sigma-Aldrich.

Cell proliferation assay

TF-1 cells stably expressing ABL, ABL$^{\triangle 45}$, or BCR-ABL were constructed using lentivirus. These stable TF-1 cell lines were seeded at a concentration of 2×10^4 cells/well or 1×10^5 cells/well in each well of a 12-well plate and cultured with or without GM-CSF. Cell numbers were counted daily for 7 days.

Protein degradation analysis

Cells transfected with plasmids bearing ABL derivatives were treated with 100 μg/ml cycloheximide to turn off protein synthesis. Samples were collected at the indicated time points and lysed in lysis buffer supplemented with protease inhibitors (Sigma). Samples were resolved by SDS-PAGE and analyzed by Western blotting with relevant antibodies to detect ABL derivatives or GFP. The stable protein GAPDH served as a loading control to ensure that similar amounts of inputs were used.

Detection of ubiquitylated ABL

HEK-293 cells were transfected with different combinations of expression vectors for FLAG-tagged ABL derivatives and Myc-tagged Smurf1, as indicated. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 2 mM EDTA, 50 mM NaF, and protease inhibitors). Protein extracts were immunoprecipitated using an anti-FLAG M2 affinity gel (Sigma). To reduce noncovalent ABL-binding proteins, immunoprecipitates were washed three times with SDS-containing buffer (0.1% SDS plus lysis buffer). Ubiquitylated ABL alleles were detected by Western blotting analysis using ubiquitin antibody.

EdU assay

TF-1 cells stably expressing ABL derivatives were seeded in a 6-well plate and cultured with GM-CSF for 24 h. Then 50 μM EdU was added in the medium, followed by incubation for 2 h. The EdU incorporation assay was performed according to the manufacturer's protocol, using EdU detection kits (RiboBio Co., Ltd., Guangzhou, China). The nuclei were observed under an Olympus IX71 inverted fluorescence microscope and analyzed with ImageJ software.

Apoptosis assay

Cell death was measured using an annexin V-allophycocyanin/propidium iodide apoptosis detection kit (KeyGen Biotech Co., Ltd., Jiangsu, China). TF-1 cells bearing various ABL alleles

were seeded in a 6-well plate and cultured without GM-CSF for 48 h. Cells were collected and washed with PBS. Then the pellets were dissolved in a master mix containing reaction buffer, annexin V-allophycocyanin, and propidium iodide at room temperature for 15 min. Samples were analyzed with an Accuri C6 Plus flow cytometer (BD).

Xenograft tumor analysis

A total of 2×10^7 TF1 cells stably expressing BCR-ABL, $ABL^{\triangle 45}$, ABL, or empty vector were suspended in PBS with 10% Matrigel and then injected into the flanks of each of six 4-week-old female BALB/c-*nu* athymic nude mice (SLAC Laboratory Animal Co. Ltd., Hunan, China). Subcutaneous tumor formation was observed and tumor size was measured with a Vernier caliper. The tumor volumes were calculated with the following formula: length × width × width × 0.5. All animal experiments were performed in accordance with the regulations for animal care and use (Nanchang University), as approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University.

Data availability statement

All relevant data are included in this manuscript.

Author contributions—Z. Y., K. S., D. M., and J. L. data curation; Z. Y., K. S., D. M., J. L., S. L., and H. R. formal analysis; Z. Y. and K. S. validation; Z. Y. investigation; Z. Y. methodology; Z. Y. writing-original draft; K. S., D. M., J. L., S. L., and H. R. writing-review and editing; S. L. and H. R. supervision; S. L. and H. R. funding acquisition; S. L. and H. R. project administration; H. R. conceptualization.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: Smurf1, SMAD-specific E3 ubiquitin protein ligase 1; GM-CSF, granulocyte/macrophage colony-stimulating factor; EdU, 5-ethynyl-2-deoxyuridine.

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