

RAPSYN-Mediated Neddylation of BCR-ABL Alternatively Determines the Fate of Philadelphia Chromosome-positive Leukemia

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Mengya Zhao, Beiying Dai, Xiaodong Li, Yixin Zhang, Chun Qiao, Yaru Qin, Zhao Li, Qingmei Li, Shuzhen Wang , Yong Yang , Yijun Chen 

Laboratory of Chemical Biology, School of Life Science and Technology, China Pharmaceutical University, Nanjing, 211198, China • State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, 211198, China • Department of Hematology, The First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital, Nanjing, 210029, China • Chongqing Innovation Institute of China Pharmaceutical University, Chongqing 401135, China

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Abstract

Philadelphia chromosome-positive (Ph⁺) leukemia is a fatal hematological malignancy. Although standard treatments with tyrosine kinase inhibitors (TKI) have achieved remarkable success in prolonging patient survival, intolerance, relapse and TKI resistance remain serious issues for patients with Ph⁺ leukemia. Here, we report a new leukemogenic process in which RAPSYN and BCR-ABL co-occur in Ph⁺ leukemia, and RAPSYN mediates the neddylation of BCR-ABL. Consequently, neddylated BCR-ABL enhances the stability by competing its c-CBL-mediated degradation. Furthermore, SRC phosphorylates RAPSYN to activate its NEDD8 E3 ligase activity, promoting BCR-ABL stabilization and disease progression. Moreover, in contrast to *in vivo* ineffectiveness of PROTAC-based degraders, depletion of RAPSYN expression or its ligase activity decreased BCR-ABL stability and, in turn, inhibited tumor formation and growth. Collectively, these findings represent an alternative to tyrosine kinase activity for the oncoprotein and leukemogenic cells and generate a rationale of targeting RAPSYN-mediated BCR-ABL neddylation for the treatment of Ph⁺ leukemia.

eLife assessment

Zhao et al describe a novel function for RAPSYN in bcr-abl fusion associated leukemia. They show that RAPSYN stabilizes the oncogenic BCR-ABL fusion protein. Their findings are **important** and the strength of findings is **convincing**. The reviewers have made suggestions about further strengthening the rigor by use of more primary samples.

Philadelphia chromosome-positive (Ph⁺) leukemia is a myeloproliferative neoplasm characterized by the reciprocal translocation between the long arms of chromosome 9 and 22, t(9;22)(q34.1;q11.2) (de Klein et al., 1982 [↗](#); Deininger et al., 2000 [↗](#)). This cytogenetic abnormality results in a *BCR-ABL* fusion gene, which encodes the chimeric protein BCR-ABL with enhanced tyrosine kinase activity (Cortes et al., 2021 [↗](#)). Based on its oncogenic role in Ph⁺ leukemia, BCR-ABL has been regarded as the most pivotal target for Ph⁺ leukemia therapy, especially for chronic myeloid leukemia (CML). Tyrosine kinase inhibitors (TKI) have been the main treatment option for Ph⁺ leukemia, remarkably prolonging the patients' life span and improving their quality of life (Hochhaus et al., 2020 [↗](#); Jabbour and Kantarjian, 2020 [↗](#)). However, most patients develop TKI resistance and relapse after long-term treatment (Braun et al., 2020 [↗](#)). It is worth noting that the increase of BCR-ABL expression can affect the sensitivity to TKIs and eventually determine the rate of TKI resistance in patients with Ph⁺ leukemia in addition to the mutations in the kinase domain (Jabbour et al., 2007 [↗](#)). Mutations in the kinase domain can change the conformation of BCR-ABL, thus interfering with the binding between TKIs and BCR-ABL and resulting in decreased therapeutic efficacy (Lussana et al., 2018 [↗](#)). In parallel, the increase of BCR-ABL expression can affect the sensitivity to TKIs and eventually determine the rate of disease progression and TKI resistance in patients with Ph⁺ leukemia (Barnes et al., 2005 [↗](#)). Therefore, effective degradation of BCR-ABL could address the issues on TKI resistance and leukemia-initiating cells (LICs), and PROTAC-based protein degradation strategy may represent a new therapeutic approach (Bekes et al., 2022 [↗](#)). Currently, based on different ubiquitin E3 ligases, including VHL, CRBN and IAP, PROTAC-based degraders at nM level have shown significant degradation of BCR-ABL in CML cell lines, cell lines carrying mutations in BCR-ABL as well as patient-derived primary cells containing multiple BCR-ABL mutations (Demizu et al., 2016 [↗](#); Lai et al., 2016 [↗](#); Shimokawa et al., 2017 [↗](#); Zhao et al., 2019 [↗](#); Burslem et al., 2019 [↗](#); Liu et al., 2022 [↗](#)). Unfortunately, the excellent cellular activity by the PROTAC-based degraders has not been able to translate to *in vivo* efficacy, even in rare examples of xenografted mouse models (Zhao, Ren et al., 2019; Jiang et al., 2021a [↗](#)), resulting in uncertain usefulness of these degraders. Nonetheless, the unsatisfactory results are not really surprising because the underlying mechanism of elevated BCR-ABL expression remains largely unclear.

Receptor-associated protein of the synapse (RAPSIN) has been identified as a classic synaptic adaptor protein that binds to the acetylcholine receptor (AChR) and several cytoskeleton-associated proteins, contributing to AChR clustering and neuromuscular junction (NMJ) formation (Huh and Fuhrer, 2002 [↗](#); Witzemann et al., 2013 [↗](#)). Later, RAPSIN was found to exert NEDD8 E3 ligase activity to catalyze the neddylation for AChR aggregation (Li et al., 2016 [↗](#)). Despite the extensive studies of RAPSIN in muscular and neuronal cells and tissues (Legay and Mei, 2017 [↗](#); Li et al., 2018 [↗](#)), with regard to its involvement in leukemogenesis, there is no available information thus far except for our previous finding. Previously, RAPSIN was found to be located in the cytosol of the typical Ph⁺ leukemia cell line K562 when a small molecule was used to probe its binding proteins using a proteomics approach (Wang et al., 2015 [↗](#)). Because of its newly identified E3 ligase activity for neddylation and its occurrence in the Ph⁺ leukemia cell line, we speculated that RAPSIN might contribute to Ph⁺ leukemia development through its enzymatic activity instead of only serving as a scaffolding protein.

As a type of post-translational modification (PTM), neddylation is sequentially catalyzed by the neuronal precursor cell-expressed developmentally downregulated protein 8 (NEDD8)-activating enzyme E1 (NAE1), NEDD8-conjugating enzyme E2 (UBE2M/UBC12 or UBE2F), and a substrate-specific NEDD8 E3 ligase to complete the covalent conjugation of NEDD8 to a lysine residue of its substrates (van der Veen and Ploegh, 2012 [↗](#); Enchev et al., 2015 [↗](#)). The neddylation of proteins can be reversed by deneddylases such as NEDP1. In the last two decades, accumulating evidence indicated the strong involvement of dysregulated neddylation in tumor progression,

neurodegenerative and cardiac diseases, aberrant immunoregulation and others (Ying et al., 2018 [↗](#); Zhou et al., 2019 [↗](#); Li et al., 2020 [↗](#); Yao et al., 2020 [↗](#)), which rationalizes the modulation of neddylation as a feasible therapeutic strategy.

In this study, we report that RAPSIN is highly expressed along with BCR-ABL in patients with Ph⁺ leukemia and promotes disease progression, presumably by stabilizing the BCR-ABL fusion protein *via* neddylation. The neddylation of BCR-ABL by RAPSIN subsequently competes its ubiquitination-dependent degradation to increase the stability of BCR-ABL. Additionally, the NEDD8 E3 ligase activity of RAPSIN can be substantially increased by SRC-mediated phosphorylation, leading to enhanced stability and activity of RAPSIN.

Results

High protein levels of RAPSIN promoted Ph⁺ leukemia progression

Prior to investigating the biological roles of RAPSIN in the pathogenesis of Ph⁺ leukemia, its expression at both mRNA and protein levels was analyzed. Neither a publicly available database nor our collection of patient samples and cell lines showed a significant increase in mRNA levels (Figure1-figure supplement 1A-C). The protein levels of RAPSIN were substantially elevated in the peripheral blood mononuclear cells (PBMC) of Ph⁺ CML (#8-11) and the bone marrow of ALL (#7) patient samples in comparison to that of healthy donors (#1-6), which was in a direct accordance with the expression of BCR-ABL (Figure 1A [↗](#)). This co-expression of RAPSIN and BCR-ABL was also found in Ph⁺ cell lines (Figure 1B [↗](#)), suggesting that the function of RAPSIN in Ph⁺ leukemia could be closely related to BCR-ABL.

To examine the relationship between RAPSIN and Ph⁺ leukemia progression, we first performed knockdown of its expression by using shRNAs. Marked reduction of RAPSIN levels and notable cytotoxicity were observed in all tested Ph⁺ leukemia cells transduced with RAPSIN shRNAs (Figure 1C [↗](#), Figure1-figure supplement 1D-F), indicating the dependence on the presence and expression level of BCR-ABL. Conversely, exogenous expression of RAPSIN rescued Ph⁺ leukemia cells from shRNA-generated toxicity (Figure 1D [↗](#)). Knockdown of RAPSIN also changed the phenotypes of Ph⁺ leukemia cells, including proliferation, G0/G1 cell cycle arrest and apoptosis (Figure 1E-G [↗](#), Figure1-figure supplement 1G, H).

Next, we subcutaneously implanted empty body transduced shRAPSIN#3 or K562 cells into NCG mice to establish a cell line derived xenotransplantation mouse model (Figure 1H [↗](#)). Tumor growth was significantly inhibited by RAPSIN silencing (Figure 1I, J [↗](#), Figure1-figure supplement 1I). Meanwhile, immunoblotting of tumor samples showed a notable downregulation of RAPSIN expression, along with the reduction of BCR-ABL levels (Figure 1K [↗](#)). After knockout of RAPSIN for remarkable depletion of BCR-ABL expression in K562 cells (Figure 1L [↗](#), Figure1-figure supplement 1K), these cells along with the empty vector-transduced K562 cells were intravenously injected into NCG mice to establish the leukemogenic mouse model (Figure 1H [↗](#)). Consequently, the survival of tumor-bearing mice was profoundly prolonged by the knockout of RAPSIN compared to the controls (Figure 1M [↗](#)). Altogether, our findings indicate that RAPSIN is highly expressed at protein level with the accordance to BCR-ABL in Ph⁺ leukemia and its depletion results in inhibiting the progression of Ph⁺ leukemia.

RAPSIN directly neddylated BCR-ABL

Previous reports determined that both nicotinic AChR subunit α₇ and muscarinic AChR subtypes M₂, M₃, and M₄ were involved in the cell proliferation of K562 cells (Cabadak et al., 2011 [↗](#); Önder Narin et al., 2021 [↗](#)). Furthermore, RAPSIN was found to exert its NEDD8 E3 ligase activity toward AChR in neuronal systems (Li, Cao et al., 2016). To determine whether RAPSIN functioned

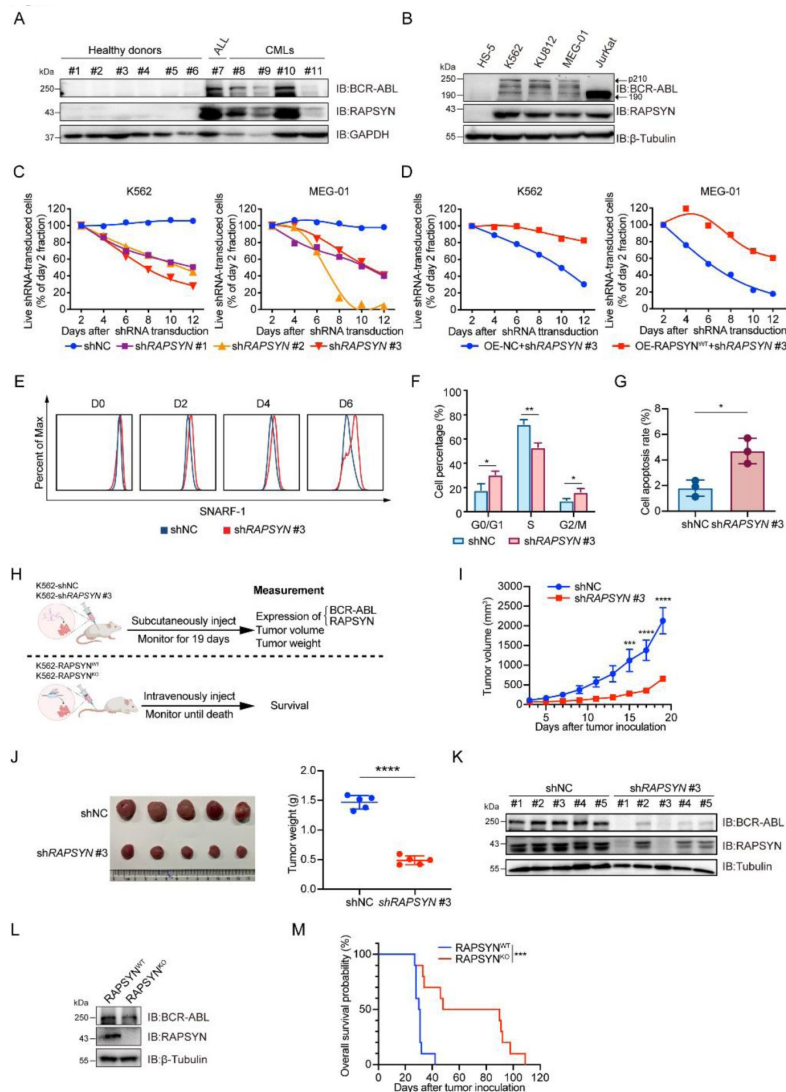


Figure 1.

High protein levels of RAPSIN promotes Ph⁺ leukemia progression.

- (A) Immunoblot of RAPSIN and BCR-ABL in the PBMCs of clinical samples.
 (B) Immunoblots of RAPSIN and BCR-ABL in Ph⁺ leukemic cells and normal bone marrow stromal cells (HS-5).
 (C) Cytotoxicity induced by shRNA-mediated RAPSIN knockdown in leukemic cells.
 (D) Rescue of leukemic cells from shRAPSIN #3-induced toxicity by exogenous expression of RAPSIN cDNA.
 (E) Analysis of SNARF-1 labeling intensity in K562 cells transduced with shNC or shRAPSIN #3.
 (F) Quantification of cell cycle analyses in K562 cells transduced with shNC or shRAPSIN #3.
 (G) Quantification of cell apoptosis analyses in K562 cells transduced with shNC or shRAPSIN #3.
 (H) An in vivo experimental design for testing the effects of RAPSIN on tumor growth and survival.
 (I) The growth curve of subcutaneous xenograft tumors was measured every two days from the third day after tumor inoculation for 19 days (five mice in each group).
 (J) Photograph and weight quantification of excised tumor xenografts from (I).
 (K) Immunoblot of RAPSIN and BCR-ABL in mouse xenograft tumor biopsies from K562 cells transduced with shRAPSIN #3 or shNC.
 (L) Immunoblot of RAPSIN and BCR-ABL in K562-RAPSIN^{WT} and K562-RAPSIN^{KO} cells.
 (M) Kaplan-Meier survival curve of NCG mice following intravenous injection of K562-RAPSIN^{WT} or K562-RAPSIN^{KO} cells, as shown in (H) (ten mice in each group).

All data represent the mean $\pm$ SD of at least three independent experiments. *P* values were calculated using unpaired Student's *t*-test or log-rank test. * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, **** *p* < 0.0001.

in a similar manner in leukemogenic cells, we investigated whether RAPSYN promoted Ph⁺ leukemia progression through neddylation of AChRs. Despite AChR subunits α_7 , M₂, M₃, and M₄ were expressed in all tested cells, no change in their neddylation was observed upon RAPSYN ablation (Figure2-figure supplement 2A, B). On the basis of the co-expression of RAPSYN and BCR-ABL, we postulated that RAPSYN could specifically mediate neddylation of BCR-ABL to promote Ph⁺ leukemia development.

To test this hypothesis, reciprocal immunoprecipitation was performed to reveal a strong interaction between RAPSYN and BCR-ABL in Ph⁺ leukemia cells (Figure 2A [↗](#), Figure2-figure supplement 2C). Similar results were obtained with exogenous expression in HEK293T cells (Figure 2B [↗](#)), further confirming the specific interaction of RAPSYN with BCR-ABL. Furthermore, GST pull-down assay with purified proteins displayed specific binding of GST-tagged RAPSYN to His-tagged BCR-ABL (Figure 2C [↗](#)), indicating that BCR-ABL is the primary target of RAPSYN-mediated neddylation. Domain mapping revealed that the $\Delta 1$ domain (1-927 aa) of BCR-ABL was responsible for the interaction with RAPSYN (Figure 2D [↗](#)).

Next, we studied whether RAPSYN could directly mediate BCR-ABL neddylation. Preeminent BCR-ABL neddylation was detected in all Ph⁺ leukemia cell lines (Figure 2E [↗](#), Figure2-figure supplement 2D). Treatment with the NAE1 inhibitor, MLN4924, significantly dampened the neddylation of BCR-ABL (Figure 2F [↗](#), Figure2-figure supplement 2E). In addition, the mutation of two glycine residues at the C-terminus of NEDD8 required for its covalent conjugating ability, or the co-expression of NEDP1 (NEDD8-specific protease 1) essentially diminished the neddylation of BCR-ABL (Figure 2G, H [↗](#)). As shown in Figure 2I [↗](#), we co-expressed either WT-RAPSYN or its C366A mutant along with BCR-ABL and NEDD8, revealing that mutation of Cys to Ala at the catalytic residue C366 significantly decreased the neddylation level of BCR-ABL. Additionally, knockout of RAPSYN abrogated BCR-ABL neddylation in the cells, and this effect was restored by transduction of RAPSYN cDNA (Figure 2J [↗](#)). These results were further corroborated by *in vitro* experiments, which showed that BCR-ABL could hardly be neddylated in the absence of RAPSYN (Figure 2K [↗](#)). Consistently, the amount of neddylated BCR-ABL was markedly reduced in tumors generated by K562 cells transfected with shRAPSYN#3 (Figure 2L [↗](#)), indicating an essential role of the ligase activity of RAPSYN in BCR-ABL neddylation.

In addition, neither overall BCR-ABL expression nor its neddylation levels were affected after the knockdown of AChRs (Figure2-figure supplement 2F). Similarly, modulation of AChR activities and their downstream PKC-RAS-ERK and JAK2-AKT signaling pathways (Kawamata et al., 2011 [↗](#); Aydin et al., 2013 [↗](#)) by either an agonist (carbachol) (Jakubik et al., 2008 [↗](#)) or antagonists (benzethonium and homatropine) (Durieux and Nietgen, 1997 [↗](#)) did not alter the expression or neddylation status of BCR-ABL (Figure2-figure supplement 2G, H).

Subsequently, we tried to identify specific modification sites on BCR-ABL. The purified proteins were used for *in vitro* neddylation reactions, and the target bands were digested with trypsin for LC-MS/MS analyses. Eight lysine residues were found to be potential NEDD8 accepting sites in BCR-ABL (Figure2-figure supplement 3). To confirm these modification sites, a series of individual Lys-to-Arg mutants were generated. Except for K257, neddylation levels of BCR-ABL at other candidate sites were all significantly reduced, confirming the modification sites of these Lys residues (Figure 2M [↗](#)).

RAPSYN attenuated c-CBL-mediated BCR-ABL ubiquitination and degradation

As decreased neddylation of BCR-ABL following either MLN4924 treatment or RAPSYN/KO was accompanied by a strong decline in its overall protein expression level (Figure 2F [↗](#), 3A-B [↗](#), Figure2-figure supplement 2E, 4), we asked whether RAPSYN-mediated BCR-ABL neddylation affects protein stability. Subsequently, the protein synthesis inhibitor CHX was applied to K562

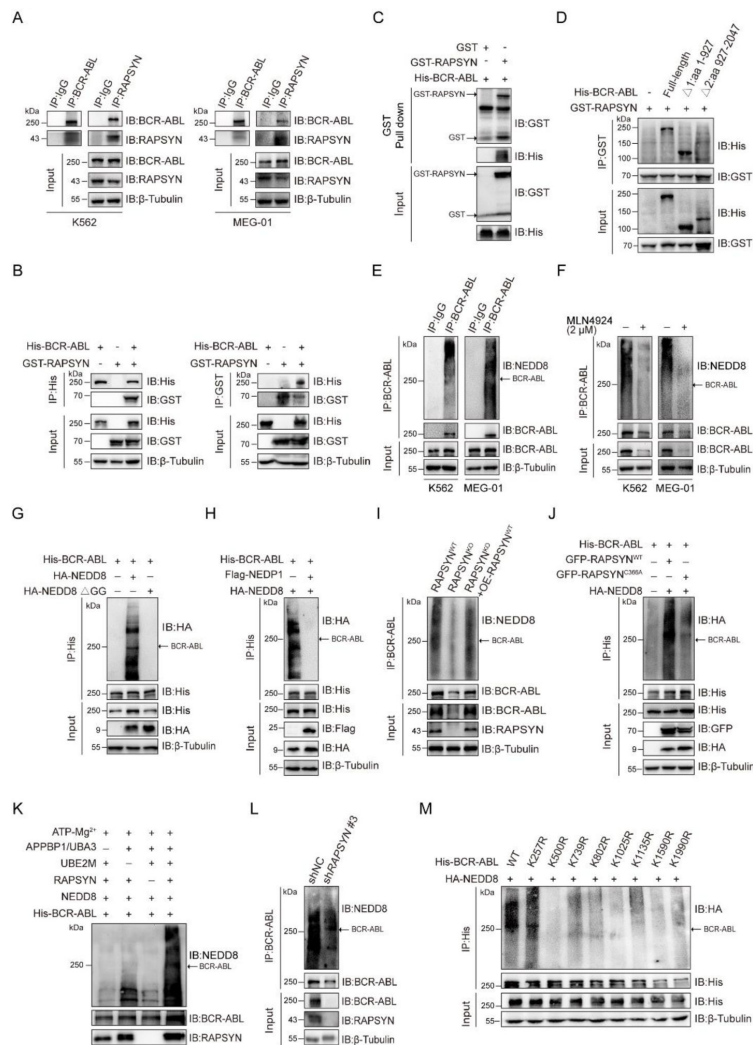


Figure 2.

RAPSYN neddylates BCR-ABL.

- (A) Co-immunoprecipitation of BCR-ABL and RAPSYN in leukemic cells.
- (B) Immunoblot of GST and His after immunoprecipitation of His or GST in HEK293T cells transfected with His-tagged BCR-ABL and GST-tagged RAPSYN.
- (C) Immunoblot of GST and His following GST pull-down after in vitro incubation of purified His-tagged BCR-ABL and GST or GST-tagged RAPSYN.
- (D) His-immunoblot of GST immunoprecipitates from HEK293T cells transfected with GST-tagged RAPSYN alone or in combination with His-tagged full-length or truncated BCR-ABL ($\Delta 1$: aa 1-927, $\Delta 2$: aa 928-2047).
- (E) Analysis of BCR-ABL neddylation levels in leukemic cells.
- (F) Analysis of BCR-ABL neddylation levels in leukemic cells treated with MLN4924 or DMSO for 24 h.
- (G) HA-immunoblot of His-immunoprecipitate from HEK293T cells transfected with His-tagged BCR-ABL and HA-tagged NEDD8 or NEDD8 Δ GG.
- (H) HA-immunoblot of the His-immunoprecipitate from HEK293T cells transfected with the indicated constructs.
- (I) Analysis of BCR-ABL neddylation levels in K562 WT, RAPSYN KO and RAPSYN KO with exogenous expression of a RAPSYN cDNA cells.
- (J) HA-immunoblot after immunoprecipitation of His in HEK293T cells transfected with His-tagged BCR-ABL, HA-tagged NEDD8, GFP-tagged WT RAPSYN or C366A RAPSYN.
- (K) Assessment of BCR-ABL neddylation by RAPSYN in vitro. Purified RAPSYN and BCR-ABL were incubated with APPBP1/UBA3, UBE2M or NEDD8 in in vitro neddylation assay.
- (L) Analysis of BCR-ABL neddylation levels in excised tumor xenografts from **Fig. 1J**.
- (M) Verification of BCR-ABL neddylation sites in HEK293T cells transfected with indicated constructs.

cells transduced with vectors encoding doxycycline-inducible *RAPSN* shRNA #3. Indeed, the expression levels of BCR-ABL declined much faster in cells with the induction of shRNA expression (**Figure 3C**). Meanwhile, we found that a clear inverse correlation between the neddylation and ubiquitination levels of BCR-ABL was observed (**Figure 3D, E**). BCR-ABL ubiquitination was remarkably reduced in the cells transfected with NEDD8 (**Figure 3F**). Consistent with these results, treatment of the cells with the proteasome inhibitor MG132 significantly increased the amount of ubiquitinated BCR-ABL accompanied by the decrease of BCR-ABL neddylation (**Figure 3G**).

To clarify the molecular basis of the battle between BCR-ABL neddylation and its ubiquitination, we detected that whether RAPSIN competes for binding to BCR-ABL with c-CBL, a reported E3 ligase mediating BCR-ABL ubiquitin-proteasome degradation (Mao et al., 2010). As a result, exogenous expression of RAPSIN interfered with the interactions between BCR-ABL and c-CBL, whereas RAPSIN ablation in K562 cells promoted c-CBL binding to BCR-ABL (**Figure 3H, I**). These data indicate that RAPSIN competes with c-CBL for binding to BCR-ABL, leading to subsequent BCR-ABL neddylation to enhance BCR-ABL stability by counteracting its proteasomal degradation.

SRC-mediated phosphorylation stabilized RAPSIN by repressing its proteasomal degradation

SRC-family protein tyrosine kinases are capable of phosphorylating RAPSIN in neuronal system, among which SRC exerts the strongest function (Mohamed and Swope, 1999). In addition, SRC has been shown to be highly expressed in primary CML cells (Yang et al., 2017). We then studied whether SRC acts as an upstream regulator to mediate RAPSIN. SRC inhibition with saracatinib or shRNA not only significantly downregulated phosphorylated (Tyr418) SRC, but also inhibited the phosphorylation of endogenous RAPSIN, resulting in a substantial decline in its protein level, whereas heterologous expression of SRC increased RAPSIN phosphorylation (**Figure 4A-C**, Figure4-figure supplement 5A). Furthermore, *in vitro* incubation with recombinant RAPSIN, SRC, and ATP resulted in strong phosphorylation of RAPSIN, which could be fully abrogated by saracatinib treatment (**Figure 4D**). LC-MS/MS analyses indicated that Tyr residues at positions 59, 152, and 336 in RAPSIN are potential phosphorylation sites by SRC (Figure4-figure supplement 5B). Then, after mutagenesis of these sites from Tyr to Phe, Y336, an evolutionarily conserved Tyr residue, was confirmed to be the primary site of RAPSIN phosphorylation (**Figure 4E**, Figure4-figure supplement 5C). As SRC has no effect on *RAPSN* mRNA levels (Figure4-figure supplement 5D, E), implying that SRC-mediated phosphorylation also affects RAPSIN stability. In fact, Ph⁺ leukemia cells were treated with saracatinib, SRC silencing or mutation of the key phosphorylation site significantly accelerated the diminishment of RAPSIN expression following CHX treatment, conversely, expressing exogenous SRC cDNA prolonged the half-life of RAPSIN (**Figure 4F-I**).

To explore the molecular mechanisms responsible for the increased stability of phosphorylated RAPSIN, Ph⁺ leukemia cells were treated with saracatinib or transduction of shSRC followed by incubation with MG132. In all circumstances, MG132 could rescue the decrease of RAPSIN induced by saracatinib treatment or shSRC knockdown (**Figure 4J, K**). Clearly, the specific phosphorylation of RAPSIN at Y336 by SRC led to its increased stability by preventing the proteasomal degradation, thereby maintaining the high levels of RAPSIN in Ph⁺ leukemia.

Phosphorylated RAPSIN potentiated its NEDD8 E3 ligase activity and promoted BCR-ABL stabilization

To dissect the role of SRC-mediated phosphorylation of RAPSIN, we tested whether phosphorylation of RAPSIN at Y336 affects its ligase activity. Immunoblotting revealed saracatinib treatment or SRC silencing reduced BCR-ABL neddylation and its protein expression, while exogenous expression of SRC cDNA strongly increased it (**Figure 5A-C**). Additionally, co-

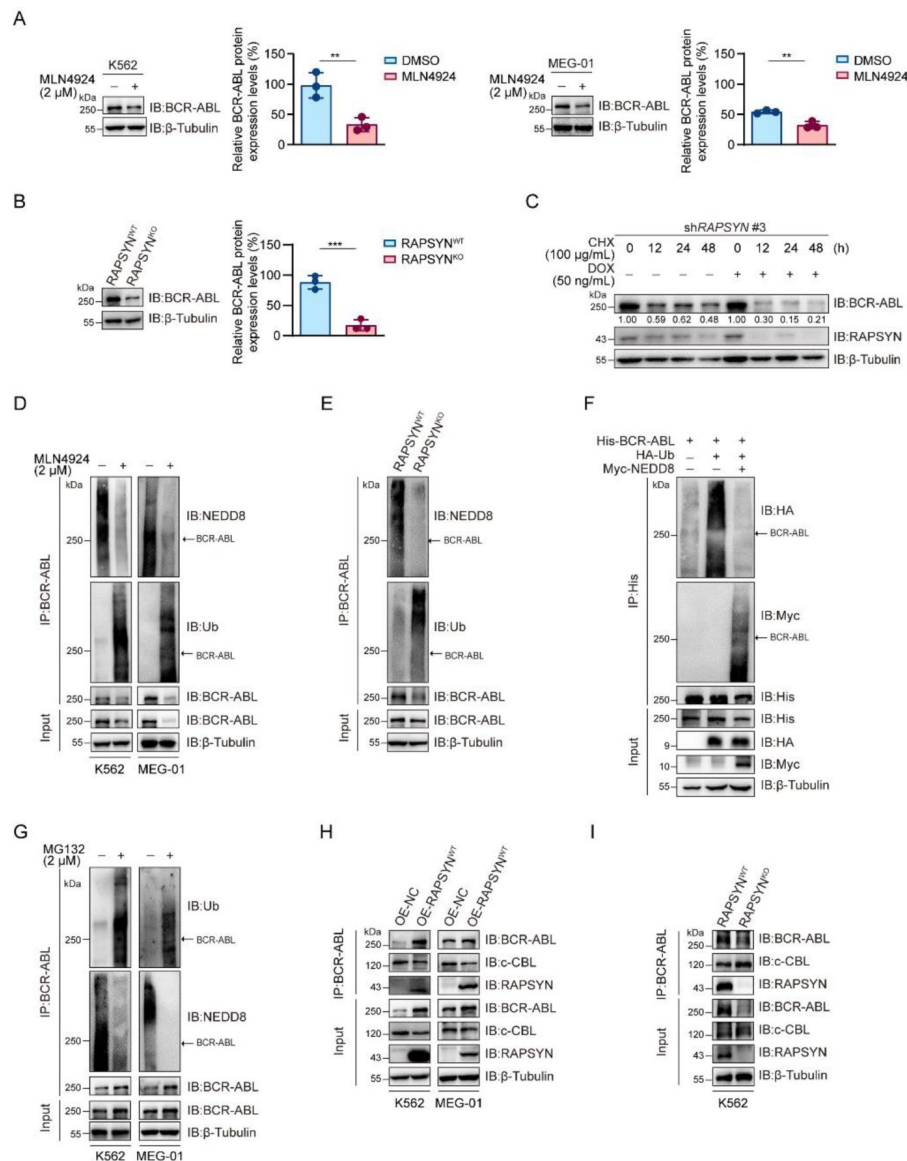


Figure 3.

RAPSIN attenuates BCR-ABL ubiquitination and degradation.

- (A) Immunoblot of BCR-ABL in leukemic cells treated with MLN4924 or DMSO for 24 h and corresponding quantification of three independent replicates.
- (B) Immunoblot of BCR-ABL in K562 WT and RAPSIN KO cells and corresponding quantification of three independent replicates.
- (C) Assessment of BCR-ABL protein stability in K562 cells expressing DOX-inducible shRAPSIN #3 treated with CHX alone or in combination with DOX at indicated time points by immunoblotting.
- (D) Analysis of BCR-ABL neddylation and ubiquitination levels in leukemic cells treated with MLN4924 or DMSO for 24 h.
- (E) Analysis of BCR-ABL neddylation and ubiquitination levels in K562 WT and RAPSIN KO cells.
- (F) Immunoblot of HA and Myc after His-immunoprecipitation in HEK293T cells transfected with His-tagged BCR-ABL, HA-tagged Ub or without Myc-tagged NEDD8.
- (G) Analysis of BCR-ABL ubiquitination and neddylation in leukemic cells treated with MG132 or DMSO for 12 h.
- (H) Co-immunoprecipitation of BCR-ABL, c-CBL and RAPSIN in leukemic cells expressing exogenous RAPSIN cDNA or empty vector.
- (I) Co-immunoprecipitation of BCR-ABL, c-CBL and RAPSIN in K562 WT and RAPSIN KO cells.

All data represent the mean $\pm$ SD of at least three independent experiments. P values were calculated using unpaired Student's t-test. ** $p < 0.01$, *** $p < 0.001$.

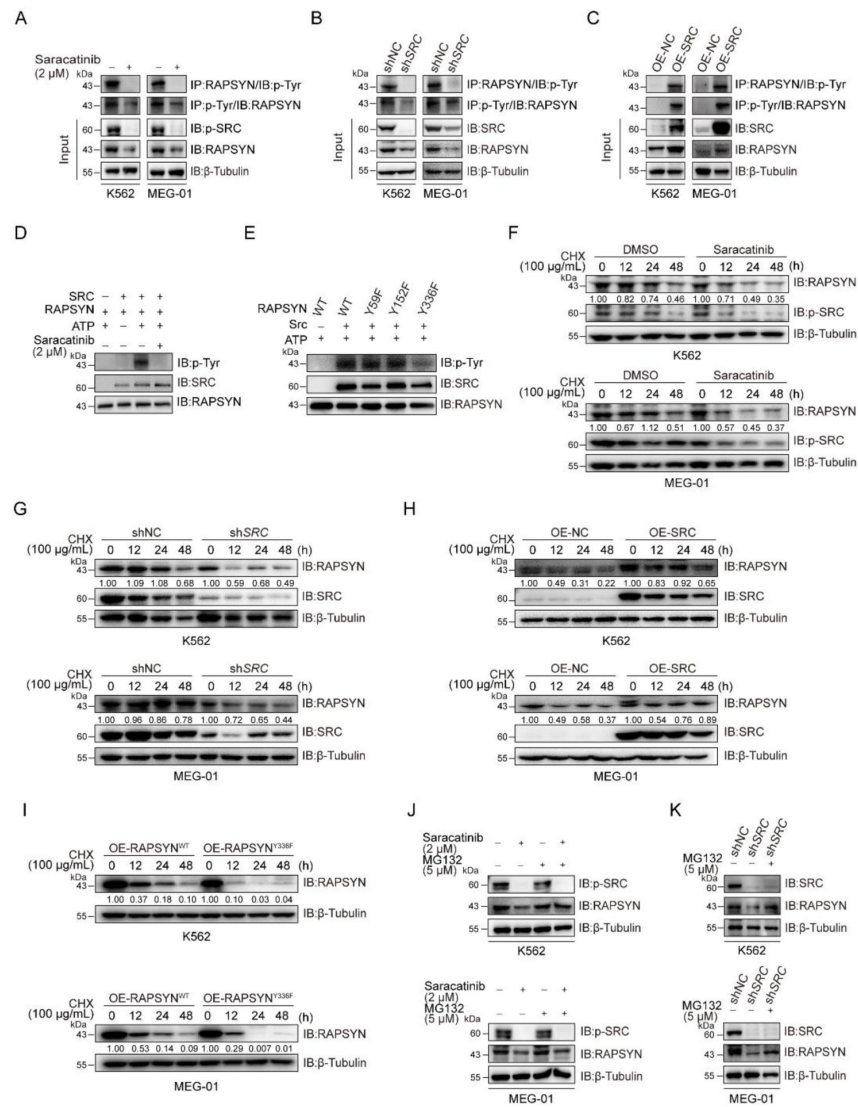


Figure 4.

SRC-mediated phosphorylation at Y336 promotes RAPSIN stability by repressing its proteasomal degradation.

- (A) Assessment of RAPSIN phosphorylation levels in leukemic cells treated with saracatinib or DMSO for 24 h.
 (B) Assessment of RAPSIN phosphorylation levels in leukemic cells transduced with shSRC or shNC.
 (C) Assessment of RAPSIN phosphorylation levels in leukemic cells expressing exogenous SRC cDNA or empty vector.
 (D) Assessment of RAPSIN phosphorylation by SRC in vitro. Purified RAPSIN and SRC were incubated with ATP in the presence or absence of saracatinib for phosphorylation assay.
 (E) Verification of RAPSIN phosphorylation sites. Purified SRC and RAPSIN WT or indicated mutants were incubated with ATP for phosphorylation assay.
 (F) Assessment of RAPSIN protein stability in leukemic cells treated with CHX in combination with saracatinib or DMSO at indicated time points by immunoblotting.
 (G) Assessment of RAPSIN protein stability in leukemic cells transduced with shSRC or shNC by immunoblotting.
 (H) Assessment of RAPSIN protein stability in leukemic cells transduced with exogenous SRC cDNA or empty vector by immunoblotting.
 (I) Assessment of RAPSIN protein stability in leukemic cells transduced with exogenous RAPSIN WT or Y336F cDNA by immunoblotting.
 (J) Immunoblot of RAPSIN in leukemic cells treated with saracatinib or DMSO for 12 h, and subsequently with MG132 or DMSO for another 12 h.
 (K) Immunoblot of RAPSIN in leukemic cells transduced with shNC or shSRC and treated with MG132 or DMSO for 12 h.

expression in HEK293T cells showed that Y336F mutation had no impact on BCR-ABL neddylation compared to co-transfection with SRC (**Figure 5D**). These results were supported by stronger neddylation of endogenous BCR-ABL in cells overexpressing WT RAPSIN, but not in the Y336F mutant (**Figure 5E**). Furthermore, protein turnover rates of BCR-ABL were determined in Ph⁺ leukemia cells and the cells with exogenous *RAPSIN*^{Y336F} expression displayed larger decrease in BCR-ABL level than those expressing *RAPSIN*^{WT} (**Figure 5F**). Therefore, RAPSIN phosphorylation at Y336 by SRC was a major contributing factor to its NEDD8 E3 ligase activity and BCR-ABL stability in Ph⁺ leukemia cells.

Phosphorylation of RAPSIN at Y336

promoted Ph⁺ leukemia progression

To assess the extent to which SRC-mediated phosphorylation of RAPSIN at Y336 contributes to the enhanced viability of RAPSIN-dependent Ph⁺ leukemia cells, we first identified a specific shSRC by screening five candidates using toxicity tests and then performing rescue experiments with SRC cDNA. Toxicity tests revealed that, albeit to varying degrees, shSRC#2, #4, and #5 induced cytotoxicity in all Ph⁺ leukemia cell lines (**Figure 6A**, Figure6-figure supplement 6A). However, exogenous SRC cDNA expression only restored the growth of Ph⁺ leukemia cells transduced with shSRC#2 (**Figure 6B**, Figure6-figure supplement 6B, C).

Subsequently, we performed rescue experiments by introducing *RAPSIN*^{WT/Y336F} cDNA or an empty vector into shSRC#2-transduced Ph⁺ leukemia cells and found that virtually complete *RAPSIN*^{WT}-induced rescue was detected in both cell lines, but *RAPSIN*^{Y336F} exhibited no restoring effect (**Figure 6C, D**). In addition, transduction of *RAPSIN*^{WT} cDNA conferred an increased resistance against saracatinib treatment, whereas the expression of *RAPSIN*^{Y336F} cDNA did not affect the drug sensitivity of the cells (**Figure 6E, F**). Furthermore, knockdown of RAPSIN substantially sensitized both cell lines to saracatinib (**Figure 6G**).

In animal models, while the overall survival of mice intravenously injected with K562 cells expressing control empty vector was significantly improved by either saracatinib administration or shRNA-mediated SRC inhibition, overexpression of WT RAPSIN fully counteracted these effects and shortened the lifespan of mice to the levels comparable to those of mice injected with K562 cells without SRC inhibition. In contrast, expression of exogenous *RAPSIN*^{Y336F} attenuated the protective effects of SRC inhibition to a much lesser extent (**Figure 6H-J**). Taken together, these results suggest that phosphorylation at Y336 by SRC is a major event in the pro-leukemogenic functions of RAPSIN in Ph⁺ leukemia development.

Discussion

In this study, we identified a novel role of RAPSIN in hematology. Indeed, RAPSIN inhibition significantly suppressed the survival of Ph⁺ leukemia. This phenotype was found to be linked to the NEDD8 E3 ligase activity of RAPSIN, which mediated BCR-ABL neddylation to enhance its stability for promoting leukemogenesis (**Figure 7**).

Balanced protein synthesis and degradation are pivotal for maintaining protein homeostasis and normal cellular function. Currently, a variety of degraders for BCR-ABL by PROTAC strategy have been reported (Demizu, Shibata et al., 2016; Lai, Toure et al., 2016; Shimokawa, Shibata et al., 2017; Zhao, Ren et al., 2019; Burslem, Schultz et al., 2019; Liu, Mi et al., 2022;), and this approach for the degradation of BCR-ABL has appeared to be effective on overcoming drug-resistance in various

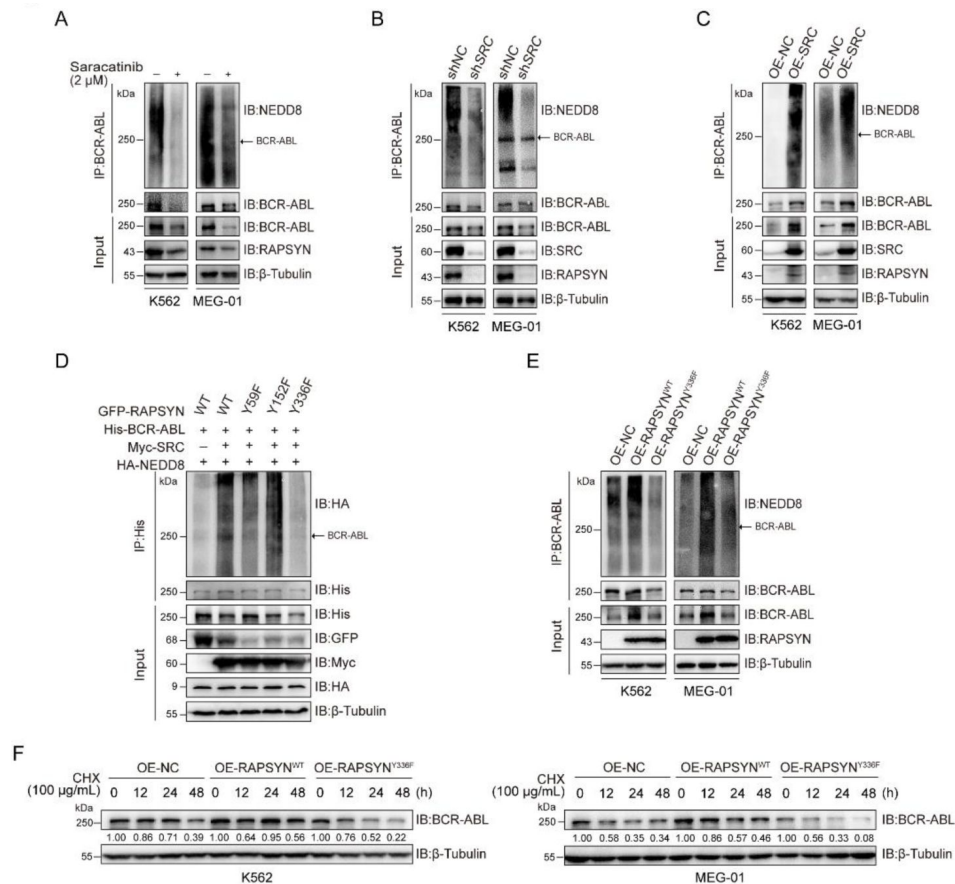


Figure 5.

RAPSYN phosphorylation at Y336 potentiates its E3 ligase activity and promotes BCR-ABL stabilization.

- (A) Immunoblot of BCR-ABL neddylation levels in leukemic cells treated with saracatinib or DMSO for 24 h.
- (B) Immunoblot of BCR-ABL neddylation levels in leukemic cells transduced with shSRC or shNC.
- (C) Immunoblot of BCR-ABL neddylation levels in leukemic cells expressing exogenous SRC cDNA or empty vector.
- (D) Effects of RAPSYN phosphorylation on BCR-ABL neddylation levels in HEK293T cells transfected with indicated constructs.
- (E) Effects of RAPSYN phosphorylation at Y336 on BCR-ABL neddylation levels in leukemic cells expressing exogenous RAPSYN WT, Y336F cDNA, or empty vector.
- (F) Assessment of BCR-ABL protein stability in leukemic cells transduced with exogenous RAPSYN WT, Y336F cDNA, or empty vector by immunoblotting.

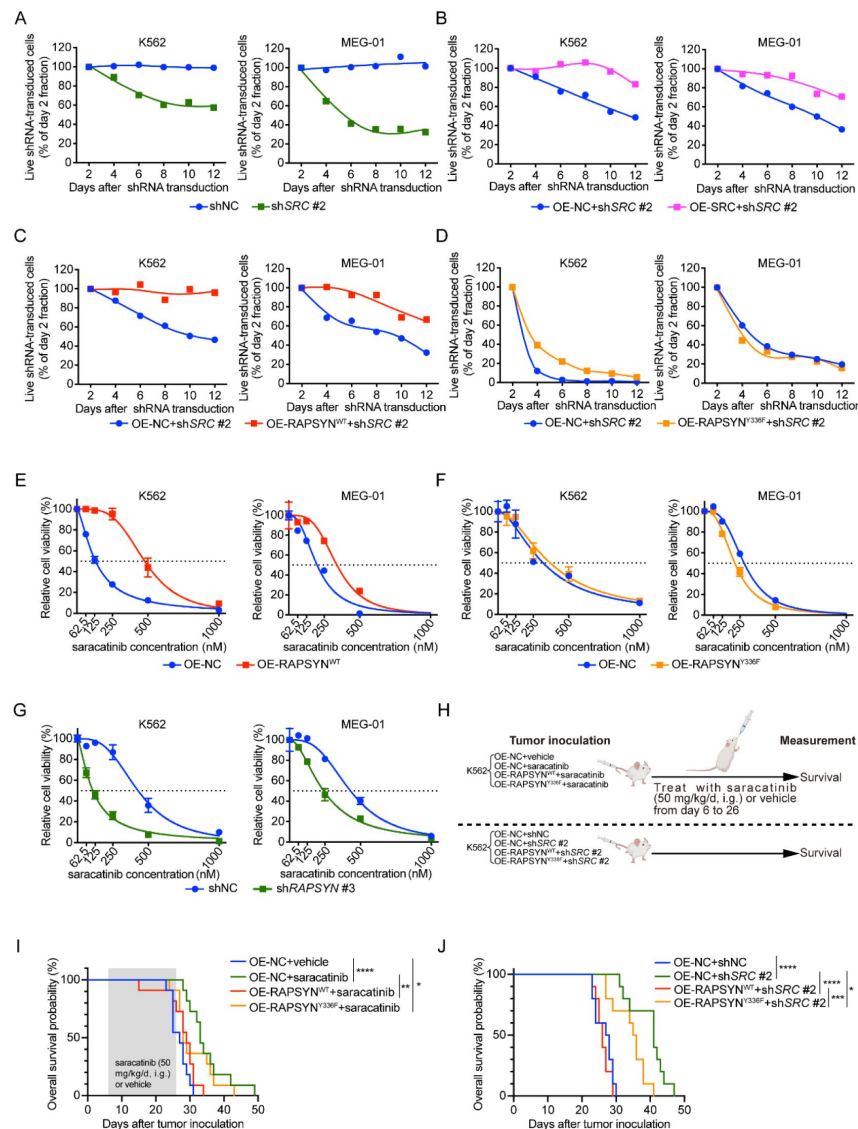


Figure 6.

SRC-mediated phosphorylation of RAPSIN at Y336 promotes Ph⁺ leukemia progression.

- (A) Cytotoxicity induced by shSRC #2-mediated SRC knockdown in leukemic cells.
 (B) Rescue of leukemic cells from shSRC #2-induced toxicity by exogenous expression of SRC cDNA.
 (C) Rescue of leukemic cells from shSRC #2-induced toxicity by exogenous expression of RAPSIN^{WT} cDNA.
 (D) Failed rescue of leukemic cells from shSRC #2-induced toxicity by exogenous expression of RAPSIN^{Y336F} cDNA.
 (E) Viability of leukemic cells transduced with either RAPSIN^{WT} cDNA or corresponding empty vector after 72 h of incubation with indicated concentrations of saracatinib.
 (F) Viability of leukemic cells transduced with either RAPSIN^{Y336F} cDNA or corresponding empty vector after 72 h of incubation with indicated concentrations of saracatinib.
 (G) Viability of leukemic cells transduced with either shNC or shRAPSYN #3 after 72 h of incubation with indicated concentrations of saracatinib.
 (H) Experimental design used to test in vivo effects of RAPSIN phosphorylation at Y336 on Ph⁺ leukemia progression and survival time.
 (I) Kaplan-Meier survival curve of NCG mice following intravenous injection of K562-RAPSYN^{WT} or K562-RAPSYN^{Y336F} cells and intragastric administration of saracatinib or corresponding vehicle from days 6 to 26 as indicated (ten mice in each group).
 (J) Kaplan-Meier survival curve of NCG mice following intravenous injection of double-transfected K562 cells (ten mice in each group). Representative results from at least three independent experiments are shown (A-G); error bars, mean ± SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; log-rank test (I-J).

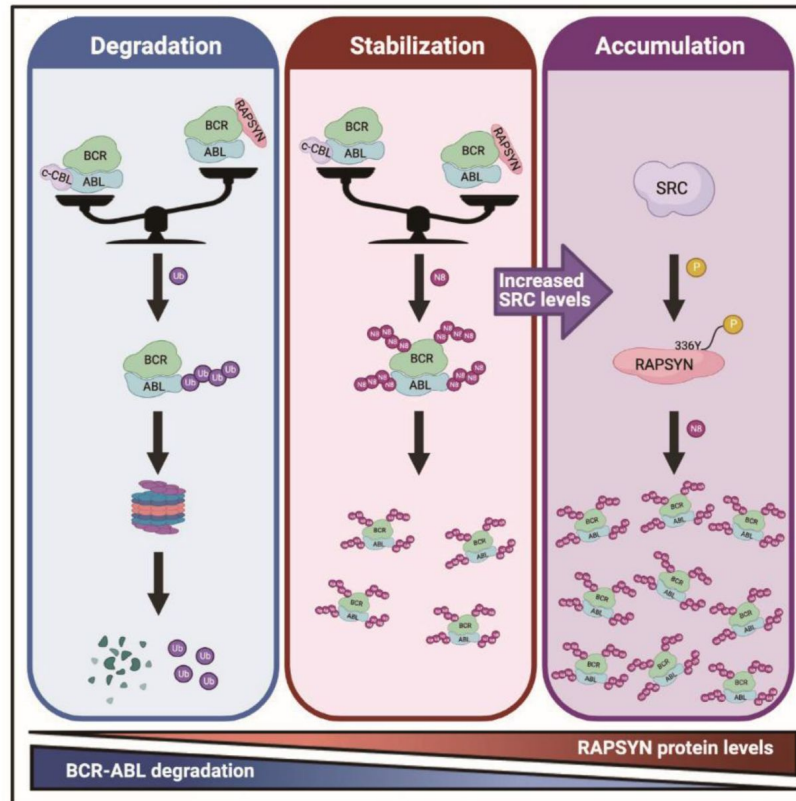


Figure.7.

Schematic representation of the competition between neddylation and ubiquitination on BCR-ABL in Ph⁺ leukemia.

unfavored physiochemical properties, significant off-target effects, various safety issues and others (Li and Song, 2020; Bekes, Langley et al., 2022). On the other hand, neddylation is a crucial type of PTM that modifies multiple Lys residues in BCR-ABL, competing with and shielding this oncoprotein from ubiquitination-mediated degradation, which provides a reasonable explanation on the poor *in vivo* efficacy of PROTAC-based degraders for BCR-ABL. Meanwhile, accumulating evidence has shown that targeting the neddylation process could be an appealing strategy for anticancer therapy (Bhalla and Fiskus., 2016; McGrail et al., 2020; Norton et al., 2021; Xie et al., 2021). MLN4924, a NEDD8-activating E1 enzyme inhibitor, has been shown to inhibit the survival of both wild-type (WT) and T315I-BCR-ABL leukemia cells as well as leukemia-initiating cells (Liu et al., 2018; Bahjat et al., 2019; Guo et al., 2019). Moreover, clinical trials of MLN4924 in combination with anticancer agents in acute myeloid leukemia (AML) have progressed to phase II (NCT03745352 and PEVENAZA [NCT04266795]) and III (PANTHER[NCT03268954] and PEVOLAM[NCT04090736]). However, the neddylation system works in a substrate- and context-dependent manner, which essentially defines its role in tumorigenesis as anti or pro, particularly relying on the substrate specificity of the NEDD8 E3 ligase. Neddylation can either facilitate ubiquitination-dependent degradation of its substrates, such as EGFR (Oved et al., 2006) and c-SRC (Lee et al., 2018), or enhance protein stability in the cases of HuR (Embade et al., 2012) and TGF- β type II receptor (Zuo et al., 2013). Thus, the antitumor effects of MLN4924 are the integrative outcome of inhibiting more *pro*- than *anti*-tumorigenic neddylation activities in the reported tumor types. Recent studies uncovered that neddylation could also inhibit tumor progression and MLN4924 stimulates tumor sphere formation and wound healing as well as promotes glycolysis (Zhou et al., 2016; Zhou, Jiang et al., 2019; Zhou and Sun, 2019). Therefore, rather than suppressing the entire neddylation system to affect a wide range of proteins, targeted inhibition of substrate-specific NEDD8 E3 ligase, such as RAPSIN, might offer a potential therapeutic opportunity for more elegant anticancer intervention with fewer side effects.

Functional studies on RAPSIN have focused on its contribution to neuromuscular transmission (Xing et al., 2019; Xing et al., 2020). To date, whether RAPSIN is involved in biological processes other than the formation and maintenance of NMJ remains poorly understood. In this study, we found that RAPSIN promoted disease progression by neddylating BCR-ABL for its resistance to c-BL-mediated proteasomal degradation. Intriguingly, although AChRs, the primary substrates of RAPSIN E3 ligase activity in neural and muscle cells, were also found to be expressed in Ph⁺ leukemia cells, they were not identified as substrates of RAPSIN for neddylation, suggesting highly orchestrated tissue-specificity of the RAPSIN enzymatic activity on its substrates.

It is well known that the generation of BCR-ABL fusion protein is a decisive characteristic of Ph⁺ leukemia (de Klein et al., 1982). Aside from the occurrence of RAPSIN in patients with Ph⁺ leukemia, the present study revealed a fascinating finding that BCR-ABL expression levels were correlated with those of RAPSIN, demonstrating the specificity of RAPSIN-mediated neddylation of BCR-ABL. Moreover, as a new type of PTM for BCR-ABL, RAPSIN-mediated neddylation was found to compete c-BL-mediated ubiquitination, causing a reduction in BCR-ABL degradation. Given the fact that the increase of BCR-ABL expression can affect the sensitivity to TKIs and eventually determine the rate of TKI resistance and LIC population in patients with Ph⁺ leukemia (Issaad et al., 2000; Barnes, Palaiologou et al., 2005), effective degradation of BCR-ABL is an alternative opportunity for the treatment. Although recent studies have greatly advanced our understanding of the regulation of BCR-ABL degradation (Burslem, Schultz et al., 2019; Shibata et al., 2020; Jiang et al., 2021b), most reported modulatory proteins are not ideal for translation to clinical settings because of their pivotal roles in sustaining normal hematological functions. In contrast, RAPSIN was nearly unexpressed in the blood of healthy donors. Thus, it is reasonable to expect that the inhibition of RAPSIN expression could lead to cytotoxicity in Ph⁺ leukemia with high specificity and marginal side effects. Furthermore, our present results showed that

knockdown of RAPSIN significantly increased the sensitivity of leukemia cells to saracatinib, implying that a combination of RAPSIN inhibition and TKI treatment can effectively control mutations and LIC-derived TKI resistance in Ph⁺ leukemia.

In summary, our work has uncovered the pivotal role that RAPSIN exerts its NEDD8 E3 ligase activity in neddylation and stabilizing BCR-ABL in the pathogenesis of Ph⁺ leukemia and thus delineate it as a potential novel therapeutic target for the treatment of Ph⁺ leukemia. More importantly, our results shed a light on future investigations that may help to extend to other cancer types for broadening our understanding of RAPSIN's involvement in hematology and oncology.

Materials and Methods

Human clinical samples

This study was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University (2019-SR-485.A1). Human peripheral blood samples were obtained from the remaining material utilized for routine laboratory tests at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) and derived from 21 patients with Ph⁺ chronic myeloid leukemia (CML), and six healthy volunteers. And one human bone marrow sample of Ph⁺ acute lymphoblastic leukemia patient was obtained from the remaining material utilized for routine laboratory tests at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). Peripheral blood and bone marrow mononuclear cells were isolated by density gradient centrifugation using the Ficoll® Paque Plus solution (17-1440-02, GE Healthcare).

Cell culture

K562 (female; CBP60529), MEG-01 (male; BP61104), and KU812 (male; BP60732) cells were purchased from COBIOER and cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640; KGM31800, KeyGEN BioTECH) containing 10-20% fetal bovine serum (FBS; FS301-02, TransGen Biotech) and 100 mg/mL streptomycin/penicillin (FG101-01, TransGen Biotech). HS-5 (male; CRL11882, ATCC), purchased from the American Type Culture Collection (ATCC) and HEK293T (KG405) from KeyGEN BioTECH were cultured in Dulbecco's modified Eagle's medium (DMEM; KGM12800, KeyGEN BioTECH) containing 10% FBS and 100 mg/mL streptomycin/penicillin. All cells were cultured in a humidified incubator with 5% CO₂ at 37°C. All the cell lines were authenticated using short tandem repeat matching analysis and tested negative for mycoplasma contamination.

Animal studies

Female NOD/ShiLtJGpt-Prkdcem26Cd52Il2rgem26Cd22/Gpt (NCG) mice (6-8 weeks), purchased from GemPharmatech Co., Ltd., were used for all in vivo studies. Mice were housed under specific pathogen-free conditions at 24 ± 1°C and 55 ± 5% humidity in a barrier facility with 12-h light-dark cycles. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with the approval of the Center for New Drug Evaluation and Research, China Pharmaceutical University (approval number: B20190925-1; Nanjing, China).

Apoptosis assay

A total of 1-5 × 10⁵ cells were washed with phosphate-buffered saline (PBS; 02-024-1ACS, Biological Industries) and resuspended in 100 µL of 1 × Annexin V binding buffer (E-CK-A211, Elabscience). The cell suspension was incubated with 2.5 µL of Annexin V-AF647 (E-CK-A211, Elabscience) and

2.5 μ L of propidium iodide (PI; E-CK-A211, Elabscience) for 20 min in the dark, followed by the addition of 400 μ L of $1 \times$ Annexin V binding buffer, and detection by flow cytometry (Thermo Attune NxT, MA, USA).

Cell viability assay

Cells were seeded at a density of 5,000 (K562) or 20,000 cells (MEG-01) per well in round-bottom 96-well plates and incubated with different concentrations of saracatinib (AZD0530, Selleck) or the corresponding amount of solvent for 72 h. Cells were then transferred to flat-bottom 96-well plates for the determination of cell viability using the CCK-8 Cell Counting Kit (A311-01, Vazyme) following the manufacturer's instructions. Each experiment was performed at least three times for each cell line.

Cell proliferation assay

Cells were washed twice with PBS, resuspended with a cell number of 2×10^6 in 1 mL PBS, and incubated with 2.5 μ M carboxyfluorescein succinimidyl ester (CFSE) solution (1948076, Thermo Scientific) or 5 μ M carboxylic acid, acetate, and succinimidyl ester (SNARF-1) solution (S-22801, Invitrogen) for 20 min at 37°C in the dark, respectively. Subsequently, 1 mL of FBS was added to stop the staining, and the cells were washed twice with complete media. Cell division was monitored by measuring CFSE or SNARF-1 dilution using flow cytometry via channels BL1 and YL1.

Cell cycle analysis

A total of 1×10^6 cells were washed once with pre-chilled PBS, fixed in ice-cold 70% ethanol, vortexing and kept for at least 20 min at -20°C . The fixed cells were washed twice with PBS and stained with 20 $\mu\text{g/mL}$ propidium iodide (PI) containing 100 mg/mL RNase A (740505, MACHEREY-NAGEL) for 15 min at room temperature. Stained nuclei were analyzed by flow cytometry and quantified using FlowJo software (BD Biosciences, NJ, USA).

Cell transfection and viral transduction

Transfection of the indicated DNA plasmids into HEK293T cells was performed using Lipofectamine 2000 (11668500, Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, transfection of HEK293T was performed when cell confluency reached 60–70%. Plasmids and Lipofectamine 2000 reagent were diluted in Opti-MEM medium (31985-070, Thermo Fisher Scientific) and incubated for 5 min at room temperature. They were then mixed together, incubated for another 20 min at room temperature, and added to the target cells. The transfected cells were collected after 48 h for further analysis.

GST pull-down assay

Purified GST or GST-RAPSYN (GenBank: NM_005055.5; 1236 bp ORF sequence) proteins were incubated with glutathione beads 4FF (SA010010, Smart-Lifescience) overnight at 4°C in binding buffer (0.14 M NaCl, 2.68 mM KCl, 2 mM KH_2PO_4 , 0.01 M Na_2HPO_4 , 10 mM DTT, pH 7.4), respectively, and incubated with purified His-BCR-ABL (p210 BCR-ABL (b3a2); 6126 bp ORF sequence) protein for another 4 h at 4°C. Beads were washed three times with washing buffer (0.14 M NaCl, 2.68 mM KCl, 2 mM KH_2PO_4 , 0.01 M Na_2HPO_4 , 0.5 mM reduced GSH, 10 mM DTT, pH 7.4), eluted with elution buffer (0.14 M NaCl, 2.68 mM KCl, 2 mM KH_2PO_4 , 0.01 M Na_2HPO_4 , 10 mM reduced GSH, 10 mM DTT, pH 7.4), and subjected to immunoblotting detection.

Immunoblotting

The cells were lysed on ice with Nonidet® P-40 (NP-40) lysis buffer (150 mM NaCl, 100 mM NaF, 50 mM Tris-HCl (pH 7.6), and 0.5% NP-40) supplemented with a protease inhibitor cocktail (78446, Thermo Fisher Scientific). Lysates were centrifuged, quantified, subjected to sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes using a Bio-Rad transfer apparatus. Membranes were blocked with 5% non-fat milk in Tris-buffered saline buffer containing 0.1% Tween-20 (TBST) at 20-25°C for 2 h, followed by incubation with primary antibody overnight at 4°C. The membranes were then washed three times in TBST buffer and incubated with species-specific HRP-conjugated secondary antibodies for 2 h at room temperature. Afterwards, the membranes were washed three times in TBST buffer, developed using the enhanced chemiluminescence (ECL) reagent, and exposed to the ChemiDoc Imaging System (Tanon, Shanghai, China).

Immunoprecipitation

Immunoprecipitation assays were performed in accordance with the manufacturer's instructions. Briefly, the cells were lysed on ice with NP-40 lysis buffer supplemented with a protease inhibitor cocktail. Cell lysates were centrifuged, quantified, and incubated with the appropriate primary antibody overnight at 4°C, and subsequently with protein A agarose beads (16-125, Millipore) for another 4 h at 4°C. Agarose was washed three times with lysis buffer and eluted with SDS-PAGE loading buffer. The eluted immunocomplexes were separated by SDS-PAGE and transferred to PVDF membranes, which were probed with primary and corresponding secondary antibodies, washed three times in TBST buffer, developed using the ECL reagent, and exposed to the ChemiDoc Imaging System.

In vitro neddylation assay

A 30 μ L reaction mixture containing 2 mM ATP-Mg²⁺ (B-20, R&D), 50 ng E1 (APPBP1/UBA3; E-313-25, R&D), 400 ng E2 (UBE2M; E2-656-100, R&D), 0.25 μ g NEDD8 (UL-812-500, R&D), 0.35 μ g His-BCR-ABL, with or without 4.77 μ g RAPSIN was incubated at 37°C for 4 h. Reaction was terminated with SDS-PAGE loading buffer and assayed using immunoblotting.

In vitro phosphorylation assay

A 40 μ L reaction mixture containing 2 mM ATP-Mg²⁺, 3 μ g RAPSIN, and 1 μ g SRC (GenBank: NM_005417.5; 1608 bp ORF sequence) protein, with or without 2 μ M saracatinib (AZD0530, Selleck), was incubated at 30°C for 30 min. Reaction was terminated with SDS-PAGE loading buffer and assayed using immunoblotting.

Animal experiments with mouse models

Female NCG mice aged 6-8 weeks were used in all the animal experiments. In the subcutaneous tumor experiment shown in **Figure 1G**, 20 mice were randomly divided into two groups, followed by subcutaneous injection of 1×10⁶ K562-shNC or K562-shRAPSIN #3 cells in 60 μ L Matrigel (354234, Corning) into the right foreleg. Tumor size was measured every two days using a digital caliper. The tumor volume was quantified using the following equation: tumor volume = 0.5 × (long diameter) × (short diameter)². When the average volume of the control group exceeded 2,000 mm³, the mice were sacrificed. The tumors were separated, and their weights were measured. As shown in the survival experiment in **Figure 1J**, NCG mice were inoculated with K562-RAPSINWT or K562-RAPSINKO (1×10⁷ cells/mouse) via the tail vein. The survival time was recorded until the mice died. As shown in the survival experiment of **Figure 6I**, 40 mice were randomly divided into 4 groups and intravenously inoculated with K562-OE-NC (20 mice), K562-OE-RAPSINWT (10 mice), or K562-OE-RAPSINY336F (10 mice). From day 6 to 26 after tumor inoculation, 10 mice inoculated with K562-OE-NC were treated with vehicle orally, while the other 30 mice inoculated with K562-OE-NC, K562-OE-RAPSINWT, or K562-OE-RAPSINY336F, 10 mice in each group, were administered saracatinib orally (50 mg/kg/d). The survival time was recorded until the mice died. In the survival experiment shown in **Figure 6J**, 40 mice were randomly divided into four groups and intravenously inoculated with double-transfected K562 cells, as indicated. The survival time was recorded until the mice died.

Identification of modification sites

To determine which lysines in BCR-ABL were neddylated using NEDD8, a neddylation reaction (50 μ L) was performed. After incubation at 37°C for 4 h, the reaction was run on an SDS-PAGE gel, silver-stained bands were excised and sent to BiotechPack Scientific Co., Ltd (Beijing, China) for liquid chromatography-mass spectrometry (LC-MS/MS) analysis. To determine which tyrosines in RAPSYN were phosphorylated by SRC, a phosphorylation reaction (50 μ L) was performed. After a 30 min reaction was run on an SDS-PAGE, silver-stained bands were excised and sent to Applied Protein Technology Co., Ltd. (Shanghai, China) for liquid chromatography-mass spectrometry (LC-MS/MS) analysis.

Plasmid construction

Eukaryotic expression vectors encoding His-, GST-, HA-, Myc-, or Flag-tagged proteins were generated by inserting PCR-amplified fragments into the pcDNA3.1(+) mammalian expression vector (V79020, Invitrogen). Eukaryotic expression vectors encoding green fluorescent protein (GFP)-tagged proteins were generated by inserting PCR-amplified fragments into the pd1-EGFP-N1 vector (6073-1, Clontech). Prokaryotic plasmids encoding GST-fusion proteins were constructed using the pGEX-4T-1 bacterial expression vector (27-4580-01, Addgene). Mutants of His-, HA-GST-, or GFP-tagged proteins were generated using the QuickMutation Site-Directed Mutagenesis Kit (D0206, Beyotime) according to the manufacturer's instructions. Briefly, whole plasmid DNA was amplified by polymerase chain reaction (PCR) for 20 cycles with specific mutant primers (Supplemental Table S1) using the QuickMutation site-directed mutagenesis kit. Next, 1 μ L DpnI was directly added to the PCR reaction mixture, followed by incubation at 37°C for 30 min and transformation. To verify the mutation sites, single clones were selected for DNA sequencing.

Preparation of stable K562 RAPSYN KO cell line

K562 RAPSYN KO cells were generated using the CRISPR/Cas9 system (Genloci Biotechnologies Inc.). RAPSYN single guide RNAs (sgRNAs) were designed using the online CRISPR design tool (<http://crispr.mit.edu/>). The sgRNA sequences were ATGGGGCGCTTCCGCGTGCTGGG, GTAGCGGCCCATCTCCGAGTGGG, and TCTGGTTGGACTGGTACAGCTGG, which were cloned into the pGK1.1/CRISPR/Cas9 vector (Genloci Biotechnologies Inc.). To obtain single clones of RAPSYN KO cells, K562 cells were transfected with the pGK1.1/CRISPR/Cas9 plasmid containing the aforementioned sgRNA sequence, expanded, selected with puromycin (0120A21, LEAGENE), and isolated by single-cell culture. Single clones obtained from RAPSYN KO cells were validated by DNA sequencing and immunoblotting.

Preparation of stable RAPSYN KD and SRC KD cell lines

Lentivirus-producing shRNA targeting either human RAPSYN or SRC mRNA was used to inhibit endogenous RAPSYN or SRC expression, respectively. All shRNAs (Supplemental Table S1) were designed using online shRNA design tools (<https://rnaidesigner.thermofisher.com> and <https://portals.broadinstitute.org>). The shRNA primers were ordered from GenScript and annealed in a thermal cycler according to the following procedure (95°C, 2 min; 85°C, 9 min; 75°C, 9 min; 65°C, 9 min; 55°C, 9 min; 45°C, 9 min; 35°C, 9 min; 25°C, 10 min; 4°C, hold) with the presence of NEBuffer 2.1 (B7202S, NEW ENGLAND BioLabs) to form a double strand with EcoRI and AgeI sticky ends. Using T4 DNA ligase (M0202L, NEW ENGLAND BioLabs), the double-stranded shRNAs were ligated into either the lentiviral backbone plasmid vector pLKO-EGFP-puro or a tet pLKO-EGFP-puro, which was digested with the restriction enzymes EcoRI-HF (R3101S, NEW ENGLAND BioLabs) and AgeI-HF (R3552S, NEW ENGLAND BioLabs). Plasmids containing shRNA or the corresponding empty vector were cotransfected with lentivirus packaging plasmids (pLP1, pLP2, and pLP/VSVG) into HEK293T cells using the linear polyethylenimine (23966, Polyscience) transfection method. After transfection for 6–8 h, the transfection reagent was replaced with a fresh medium. After incubation at 37°C, 5% CO₂ for 48 and 72 h, the resulting lentivirus supernatant was collected, respectively, and filtrated through a 0.22 μ m disc filter. Then, 15 mL of

the filtered lentivirus supernatant was concentrated through a 100 kD ultrafiltration tube at 1,500 × g and 4°C for 1 h. Ph⁺ leukemia cell lines were infected with concentrated lentivirus supernatant containing 8 µg/mL polybrene (H9268, Sigma). The culture plate or dish was centrifuged in a horizontal rotor centrifuge at 2,000 × g and 32°C for 1.5 h. After 48 h, the viral particles were replaced with fresh medium and 3 µg/mL puromycin was added for selection for another 48–72 h. Protein expression levels were analyzed by immunoblotting with anti-RAPSYN (ab118491, Abcam) or SRC (11097-1-AP, Proteintech) antibodies.

Preparation of stable RAPSYN WT, RAPSINY 336F, or SRC expression cell lines

Lentiviruses overexpressing RAPSYN WT, RAPSYN Y336F, or SRC were purchased from GenePharma. The volume of virus required was calculated using the following equation: Ph⁺ leukemia cell lines were infected using the spin-infection method described above. 48 h after infection, the viral particles were replaced with fresh medium. Stable RAPSYN WT, RAPSINY 336F, or SRC expressing cells were selected in the presence of 3 µg/mL puromycin for 48–72 h. Protein expression levels were analyzed by immunoblotting with anti-RAPSYN or SRC antibodies.

$$\text{virus volume} = \frac{\text{MOI} \times \text{cell number}}{\text{virus titer}}$$

Protein expression and purification

Recombinant pGEX-4T-1-GST-RAPSYN plasmid were transformed into the ArcticExpress (DE3) pRARE2 competent cells (AYBIO-G6023, ANGYUBIO) and treated with 0.4 mM isopropyl-β-D-thiogalactoside (367-93-1, Sangon Biotech) to induce fusion protein expression under 8°C culture condition. After 50 h, cells were harvested, resuspended in PBS (0.14 M NaCl, 2.68 mM KCl, 2mM KH₂PO₄, 0.01M Na₂HPO₄, 10 mM DTT, pH 7.4), and sonicated on ice. Precipitates were removed from the cell lysates by centrifugation. Recombinant GST-RAPSYN was purified from the supernatant by GST-affinity chromatography (SA010010, Smart-Lifescience) and size-exclusion chromatography (17-0060-01, GE Healthcare). Purified GST-RAPSYN protein was digested with thrombin (T8021, Solarbio) for 6 h at 4°C to remove the GST tag. Recombinant pcDNA3.1-His-BCR-ABL plasmids were transfected into HEK293T cells, which were collected after 48 h and lysed on ice using Nonidet P-40 (NP-40) lysis buffer with a protease inhibitor cocktail. Cell lysates were centrifuged, then the supernatant fraction incubated with the anti-BCR-ABL antibody overnight at 4°C and subsequently with protein A magnetic beads (73778, Cell Signaling Technology) for another 4 h at 4°C. The bead complexes were washed three times with washing buffer (Tris 25 mM, NaCl 0.15 M, Tween-20 0.005%, pH 7.5), eluted with elution buffer (glycine 0.1 M, pH 2.0), and mixed with neutralization buffer (Tris 1 M, pH 9.0) for neutralization.

Protein stability assay

RAPSYN^{WT}, RAPSYN^{Y336F}, or BCR-ABL-transfected Ph⁺ leukemia cells were incubated with 100 mg/mL cycloheximide (CHX, A8244; Cell Signaling Technology) for the indicated time points. Cells were harvested and lysed on ice using NP-40 lysis buffer supplemented with a protease inhibitor cocktail. The supernatant was collected and subjected to immunoblotting using anti-RAPSYN or SRC antibodies.

Quantitative reverse transcription-PCR (RT-PCR)

High-quality RNA was isolated from cells or tissues using Trizol reagent (AJF1807A, Takara) according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA using the HiScriptIIRT SuperMix for qPCR (R233-01, Vazyme). The ChamQ SYBR qPCR Master Mix (Q331-02, Vazyme) was used for two-step RT-PCR analysis on an Applied Biosystems StepOnePlus Real-Time PCR instrument. The samples were analyzed in triplicate. The expression value of the target

gene in a given sample was normalized to the corresponding expression of ACTB or GAPDH. The 2- $\Delta\Delta\text{Ct}$ method was used to calculate the relative expression of target genes. The primers used are listed in Supplemental Table S1.

Cytotoxicity assay

Lentiviruses co-expressing GFP were used to assess the toxicity of shRNAs. Flow cytometry was performed two days after shRNA transduction to determine the initial GFP-positive proportion of live cells for each shRNA. Subsequently, the cells were sampled every two days over time. The GFP-positive proportion at each time point was normalized to that of the day two. Each shRNA experiment was performed at least three times for each cell line.

Statistical analysis

All in vitro experiments were repeated at least three times. Animals were randomly assigned to different groups for each in vivo study. Kaplan-Meier survival analysis was used for all survival studies, and the log-rank test was used to determine significant differences between groups. Differences with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ were considered significant. Prism 8 (GraphPad Software, CA, USA) was used for statistical analysis. Representative results from at least three independent replicates are shown. Data are presented as the means $\pm$ SD, and significant differences were determined using the Student's t test, unpaired Student's t test, or one-way analysis of variance test.

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“Double First-Class” University Project	CPU2022QZ014	Yijun Chen
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Author contributions

Mengya Zhao, Formal analysis, Investigation, Data curation, Methodology, Writing - original draft; Beiyong Dai, Formal analysis, Investigation, Data curation, Methodology, Writing - original draft, Project administration, Funding acquisition; Xiaodong Li, Formal analysis, Investigation, Data curation, Methodology; Yixin Zhang, Formal analysis, Investigation, Data curation, Methodology; Chun Qiao, Data curation; Yaru Qin, Data curation; Qingmei Li, Data curation; Zhao Li, Data curation; Shuzhen Wang, Supervision, Project administration, Funding acquisition, Writing - review & editing; Yong Yang, Supervision, Resources; Yijun Chen, Conceptualization, Supervision, Resources, Writing - review & editing, Funding acquisition.

Competing interests

A Chinese patent application was filed with the number of 202210107464.7. China Pharmaceutical University owns the patent rights, and Y. C., Y. Y., M. Z., B. D., X. L., S. W. and X. Z. are the inventors of the patent.

Data availability

The data generated in this study are available upon request from the corresponding author.

Ethics

This study was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University (2019-SR-485.A1). All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with the approval of the Center for New Drug Evaluation and Research, China Pharmaceutical University (approval number: B20190925-1; Nanjing, China).

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Author information

Mengya Zhao

Laboratory of Chemical Biology, School of Life Science and Technology, China Pharmaceutical University, Nanjing, 211198, China
ORCID iD: [0000-0002-6339-1892](https://orcid.org/0000-0002-6339-1892)

Beiyong Dai

State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, 211198, China

Xiaodong Li

Laboratory of Chemical Biology, School of Life Science and Technology, China Pharmaceutical University, Nanjing, 211198, China

Yixin Zhang

State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, 211198, China

Chun Qiao

State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, 211198, China, Department of Hematology, The First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital, Nanjing, 210029, China

Yaru Qin

State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, 211198, China

Zhao Li

Laboratory of Chemical Biology, School of Life Science and Technology, China Pharmaceutical University, Nanjing, 211198, China

Qingmei Li

Laboratory of Chemical Biology, School of Life Science and Technology, China Pharmaceutical University, Nanjing, 211198, China

Shuzhen Wang

Laboratory of Chemical Biology, School of Life Science and Technology, China Pharmaceutical University, Nanjing, 211198, China

For correspondence: shuzhenwang@cpu.edu.cn

ORCID iD: [0000-0003-3869-2463](https://orcid.org/0000-0003-3869-2463)

Yong Yang

State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, 211198, China

For correspondence: yy@cpu.edu.cn

ORCID iD: [0000-0002-2841-8420](https://orcid.org/0000-0002-2841-8420)

Yijun Chen

Laboratory of Chemical Biology, School of Life Science and Technology, China Pharmaceutical University, Nanjing, 211198, China, State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, 211198, China, Chongqing Innovation Institute of China Pharmaceutical University, Chongqing 401135, China

For correspondence: yjchen@cpu.edu.cn

ORCID iD: [0000-0002-4920-152X](https://orcid.org/0000-0002-4920-152X)

Editors

Reviewing Editor

Amit Verma

Albert Einstein College of Medicine, United States of America

Senior Editor

Mone Zaidi

Icahn School of Medicine at Mount Sinai, United States of America

Reviewer #1 (Public Review):

The manuscript by Zhao et al describes the identification of RAPSIN, a NEDD8 E3 ligase previously studied for its role in acetylcholine receptor clustering and neuromuscular junction formation, as a factor promoting the stabilisation of the BCR-ABL oncogene in Chronic Myeloid Leukemia (CML) cells. The authors have identified that NEDDylation of BCR-ABL by RAPSIN antagonises its poly-ubiquitin and subsequent proteasome-based degradation. Knocking down RAPSIN with shRNA led to increased poly-ubiquitination and

faster turnover of BCR-ABL. Furthermore, they describe that SRC-dependent phosphorylation of RAPSIN facilitates its NEDD8-ligase activity.

The authors' findings are primarily rooted in a series of well-conducted in vitro experiments using two CML cell lines, K562 and MEG-01. While the findings are interesting and novel, further work to corroborate these findings in primary CML samples would have greatly strengthened the potential real-world relevance of these discoveries. The authors appear to have some PBMCs from primary CML patients and a BM sample from a Ph+ ALL in which they performed western blot analyses (Fig 1). Couldn't these samples have been used to at least confirm some of the key discoveries? For example, the neddylation of BCR-ABL, or; sensitivity of primary leukemic cells to RAPSIN knockdown, and/or; phosphorylation of RAPSIN by SRC?

The authors initially interrogated a fairly dated (circa 2009) microarray-based primary dataset to show that the increase in RAPSIN is primarily a post-transcriptional event, as mRNA levels are not different between healthy and CML samples. It would be interesting to see whether differences might be more readily seen in more recent RNA-seq datasets from CML patients, given the well-known differences in sensitivity between the two platforms. Additionally, I wonder if there would be transcriptional signatures of increased NEDDylation (or RAPSIN-induced NEDDylation) that could be interrogated in primary samples? Furthermore, there are proteomics datasets of CML cells made resistant to TKIs (through in vitro selection experiments) that could be interrogated for independent validation of the authors' discoveries. For example: from K562 cells, PMID: 30730747 or PMID: 34922009.

Reviewer #2 (Public Review):

In this study the authors aim to elucidate the role of RAPSIN in BCR-ABL-mediated leukemogenesis. RAPSIN is mainly known as a scaffolding protein for anchoring acetylcholine receptors (AChRs) to the cytoskeleton in muscle cells, facilitating AChR clustering through neddylation (Li et al., 2016). The authors demonstrate, through a broad and rigorous array of biochemical assays, that RAPSIN also plays a crucial role in the neddylation of BCR-ABL in leukemia cells. Their results indicate that this process shields BCR-ABL from ubiquitination and subsequent degradation, likely through a mechanism involving competition for binding with the BCR-ABL ubiquitin ligase c-CBL. In addition, the authors delve into the regulatory mechanisms underlying RAPSIN stability, demonstrating that it is enhanced through phosphorylation by SRC. This discovery further deepens our understanding of the complex dynamics of the molecular interactions that regulate BCR-ABL stability in leukemia.

To confirm the physiological significance of their findings, the authors effectively utilize cell viability assays and in vivo models. The integration of these approaches lends strength and validity to their conclusions.

The implications of the findings presented in this study are important, particularly in relation to our understanding of the pathogenesis and potential therapeutic strategies for Philadelphia chromosome-positive leukemias. By illuminating the role of RAPSIN in the regulation of BCR-ABL stability, this research potentially uncovers avenues for the development of targeted therapies, making a significant contribution to the field.

Most of the conclusions drawn in this paper are well supported by data, but some aspects of the data need to be clarified and extended:

1. The authors propose that targeting RAPSIN in Ph⁺ leukemia could have a high therapeutic index, suggesting that inhibition of RAPSIN may lead to cytotoxicity in Ph⁺ leukemia with high specificity and minimal side effects. To substantiate this assertion, the authors should investigate the impact on cell viability upon RAPSIN knockdown in non-Ph leukemic cell lines or HS-5 cells (similar to Figure 1C), despite their lower RAPSIN protein levels.
2. The authors intriguingly show that the protein levels of RAPSIN are significantly enriched in Ph⁺ patient samples and cell lines (Figure 1A,B), even though the mRNA levels remain unchanged (Supplementary Figure 1 A-C). This observation merits a clear explanation in the context of the presented results. The data in the manuscript does imply a feedforward loop mechanism (Figure 7), where BCR-ABL activates SRC, which subsequently stabilizes RAPSIN, which in turn helps protect BCR-ABL from c-CBL-mediated degradation. If this is the working hypothesis, it would be beneficial for the reader to see supporting evidence.
3. The authors present compelling evidence to suggest that RAPSIN may possess direct NEDD8-ligase activity on BCR-ABL. To strengthen this claim, it may be valuable to conduct further assays involving a ligase-deficient mutant, such as C366A, beyond its use in Figure 2J. Incorporating this mutant into the in vitro assay illustrated in Figure 2K, for instance, could offer substantial validation for the claim. In addition, showing whether the ligase-deficient mutant is capable of phenocopying the phosphorylation-mutant Y336F, as showcased in Figures 5E, F, and 6D, F, would be beneficial.
4. The observations presented in Figures 6 C-G require additional clarification. Notably, there are discrepancies in relative cell viability effects in K562 cells, and to some extent in MEG-01 cells, under conditions that are indicated as being either identical or highly similar. For instance, this inconsistency is observable when comparing the left panels of Figure 6C and 6D in the case of NC overexpression + shSRC#2, and the left panels of Figure 6E and 6G with NC overexpression or shNC, respectively. Listing potential causes of these discrepancies would strengthen the overall validity of the findings and their subsequent interpretation.
5. Throughout the manuscript, immunoblots which showcase immunoprecipitations of BCR-ABL or His-BCR-ABL depict poly-neddylation (e.g. Figures 2E-M, 3D-G, and 5A-E) and poly-ubiquitination (e.g. Figures 3D-G) patterns/smears where these patterns seem to extend below the molecular weight of BCR-ABL. To enhance clarity, it would be valuable for the authors to provide an explanation in the text or the figure legend for this observation. Is it reflective of potential degradation of BCR-ABL or is there another explanation behind it?