

Inhibition of SRC Corrects GM-CSF Hypersensitivity That Underlies Juvenile Myelomonocytic Leukemia

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Abstract

Juvenile myelomonocytic leukemia (JMML) is an aggressive myeloproliferative neoplasm in children characterized by the overproduction of monocytic cells that infiltrate the spleen, lung, and liver. JMML remains a disease for which few curative therapies are available other than myeloablative hematopoietic stem cell transplant (HSCT); however, relapse remains a major cause of treatment failure and the long-term morbidities of HSCT for survivors are substantial. A hallmark feature of JMML is acquired hypersensitivity by clonal myeloid progenitor cells to granulocyte macrophage-colony stimulating factor (GM-CSF) via a largely unknown mechanism. Here, we identify c-Cbl (henceforth referred to as Cbl) as a GM-CSF receptor (GMR) adaptor protein that targets Src for ubiquitin-mediated destruction upon GM-CSF stimulation and show that a loss of negative regulation of Src is pivotal in the hyperactivation of GMR signaling in Cbl-mutated JMML cells. Notably, dasatinib, an U.S. Food and Drug Administration–approved multikinase inhibitor that also targets Src family, dramatically attenuated the spontaneous and GM-CSF-induced hypersensitive growth phenotype of mononuclear cells from peripheral blood and bone marrow collected from JMML patients harboring Cbl or other known JMML-associated mutations. These findings reveal Src kinase as a critical oncogenic driver underlying JMML. *Cancer Res*; 73(8); 1–11. ©2013 AACR.

Introduction

The majority of juvenile myelomonocytic leukemia (JMML)–associated mutations have been mapped to genes that encode proteins that signal through the Ras/mitogen-activated protein kinase (MAPK) pathway, including *NF1*, *NRAS*, *KRAS*, and *PTPN11* (*SHP2*), which account for approximately 75% of JMML patients (1). Recently, we identified *Casitas B-lineage Lymphoma* (*CBL*) mutations that were reduced to homozygosity in leukemic cells as a result of acquired isodisomy in approximately 10–15% of children with JMML (2, 3), thereby establishing *CBL* as a new JMML tumor suppressor gene. Importantly, *CBL* mutations in JMML patient samples were mutually exclusive with respect to other Ras pathway-associated mutations but nevertheless displayed the classic granulocyte macrophage-colony stimulating factor (GM-CSF) hypersensitivity

phenotype, which suggests a possible convergence of Cbl and Ras signaling pathways. Consistent with this notion, *CBL* mutations have been confirmed in other Ras-related disorder, Noonan syndrome (4), as well as in myelodysplastic syndromes, atypical chronic myelogenous leukemia and chronic myelomonocytic leukemia, diseases that primarily occur in adults that share some clinical and molecular features with JMML (5–7).

Cbl is a RING-type E3 ubiquitin ligase that ubiquitylates receptor and non-receptor tyrosine kinases (8). Cbl contains a highly conserved N-terminal tyrosine kinase-binding (TKB) domain that mediates interaction between Cbl and phosphorylated tyrosines on activated receptor tyrosine kinases such as epidermal growth factor receptor (EGFR; ref. 9), platelet-derived growth factor receptor (PDGFR; ref. 10) and c-Kit (11), as well as non-receptor tyrosine kinases JAK2, ZAP-70, and Syk (12). The RING finger is separated from the TKB domain by an alpha helical structure known as the linker region, which is critical for the regulation of E3 function (13). Crystallographic study has revealed that the linker region contacts the TKB domain, the RING finger, and the E2 ubiquitin-conjugating enzyme (14). Y371 within the linker region becomes phosphorylated upon interaction with tyrosine kinases such as EGFR and insulin receptor, leading to conformational change in Cbl and activation of its E3 activity (13). Moreover, C-terminal to the RING finger is a proline-rich domain that mediates interaction with SH3-containing and SH2 proteins such as the p85 subunit of PI3K (15). Notably, although Cbl's E3 function serves as a negative regulator of signaling, its role as an adaptor molecule potentiates signal

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transduction (13). We and others have recently showed that JMML-associated *CBL* mutations, including the most common Y371H substitution, result in the loss of E3 function (3, 7). Although these studies have provided important insights into the possible consequences of *CBL* mutations, the mechanisms responsible for the GM-CSF hypersensitivity phenotype invariably displayed by JMML progenitor cells remains largely unknown.

Materials and Methods

Cells

HEK293 and TF-1 cells were obtained from the American Type Culture Collection. HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Wisent) at 37°C in a humidified 5% CO₂ atmosphere. TF-1 cells were maintained similarly in RPMI-1640 (Wisent) medium supplemented with 10% FBS and 2 ng/ml GM-CSF (Invitrogen).

Retroviral production and BaF3-GMR cell generation

GM-receptor α (GMR α) and GMR β c were subcloned into pRUFneo:IRES retroviral expression system (pRUFneo:IRES-GMR α / β c) and cotransfected with pEQEco packaging plasmid (both generous gifts from Dr. Tim Hercus; Centre for Cancer Biology, Adelaide, Australia) into HEK293 cells. The media was replaced with fresh media the next day. Viral supernatant was harvested 2 and 3 days posttransfection and passed through a 0.45 μ m filter (Pall). BaF3-HA-Cbl (WT, Y371H, 70Z) cells were generated as previously described (6), maintained in RPMI-1640 with 10% FBS, 10 μ g/mL mIL-3 (R&D), 10 ng/mL puromycin (Sigma), and transduced with RUFneo:IRES-GMR α / β c retrovirus with polybrene (Millipore). Cell populations stably expressing GMR were established using G418 resistance (Sigma) and confirmed by Western blot analysis. These BaF3-GMR-HA-Cbl (WT, Y371H, 70Z) cells were maintained in RPMI-1640 medium with 10% FBS, 2 ng/mL GM-CSF, 10 ng/mL puromycin, and 0.25 mg/mL G418.

Generation of shCbl and ShLyn cell lines

TF-1-shCbl and TF-1-shScr cells were generated using SMARTvector 2.0 Lentiviral shRNA particles targeted against human Cbl (Dharmacon). Cell populations with stable Cbl knockdown were selected with puromycin, sorted on a FACS Aria (BD Biosciences) and further confirmed by Western blot analysis. The stable cell lines were maintained in RPMI-1640 medium with 10% FBS, 2 ng/mL GM-CSF, and 10 ng/mL puromycin. BaF3-GMR-HA-Cbl(Y371H)-ShLyn and -shScr cells were generated using mouse lentiviral shRNA directed against Lyn kinase obtained from Dr. Jason Moffat (Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, Ontario, Canada).

Antibodies

Rabbit polyclonal antibodies against Cbl, pCblY774, Lyn, Src, phosphorylated (p)Src(Y416) as well as Src phosphorylated on Y527, JAK2, pJAK2, pSTAT5, and STAT5 were obtained from

Cell Signalling Technologies. Antibodies against GMR α , GMR β c (monoclonal and polyclonal), Cbl (monoclonal), and polyclonal extracellular signal-regulated kinase (pERK) were obtained from Santa Cruz Biotechnology. Monoclonal antibodies against HA (12CA5), pTYR (4G10), and ubiquitin were obtained from Boehringer Ingelheim, Millipore, and Dako, respectively. Monoclonal β -actin and pERK antibodies were obtained from Sigma.

Plasmids

Plasmids encoding pDEST4.0-HA-Cbl (WT, Y371H, 70Z) were generated as previously described (3). pSG5-GMR α and pSG5-GMR β c constructs were also generously provided by Dr. Tim Hercus. All constructs were verified by direct DNA sequencing.

Src knockdown via siRNA

Endogenous Src in HEK293 cells was silenced using ON-TARGETplus SMARTpool siRNA (Dharmacon) according to the manufacturer's instructions.

Immunoprecipitation and immunoblotting

Immunoprecipitation and Western blotting were done as described previously (16).

Chemicals

Dasatinib, cycloheximide, AG490, and PP2 were obtained from Sigma. MG132 was obtained from Peptides International.

Primary patient studies

Sample collection. Bone marrow or peripheral blood samples were collected from children suspected of having JMML. The UCSF Committee on Human Research approved this study. All samples were obtained with informed consent. Mononuclear cells were isolated according to standard procedures. Genomic DNA was isolated using Puregene reagents (Qiagen). Patients were genotyped for mutations in *PTPN11* exons 3 and 13, *NRAS/KRAS* exons 1 and 2, and *CBL* exons 8 and 9 according to previously reported methods (2, 17–19).

Human hematopoietic progenitor assays. In CFU-GM assays, mononuclear cells from peripheral blood or bone marrow were plated in MethoCult H4230 (StemCell Technologies) and supplemented with recombinant human GM-CSF (Peprotech). Colonies were counted after 14 days, as previously described (20). For CFU-GM assays in which cells were exposed to dasatinib (LC Laboratories) or imatinib (LC Laboratories), inhibitor was diluted in dimethyl sulfoxide (DMSO) and directly added to the MethoCult before plating.

Western blots. Mononuclear cells were rested for 30 minutes in Iscove's Modified Dulbecco's Medium and 1% bovine serum albumin followed by exposure to inhibitor for 30 minutes. Cells were stimulated with 10 ng/mL recombinant human GM-CSF (Peprotech) for 15 minutes. Resting, inhibitor exposure, and stimulation were all done at 37°C, 5% CO₂. Whole lysates were prepared, resolved on SDS-PAGE and

immunoblotted with the indicated antibodies as previously described (20).

Statistical analyses

Unpaired 2-tailed Student *t* test was used to compare between treatment groups and cell types. All statistical analysis was done using GraphPad PRISM 5.0 software. Statistical significance was achieved at the confidence limit indicated.

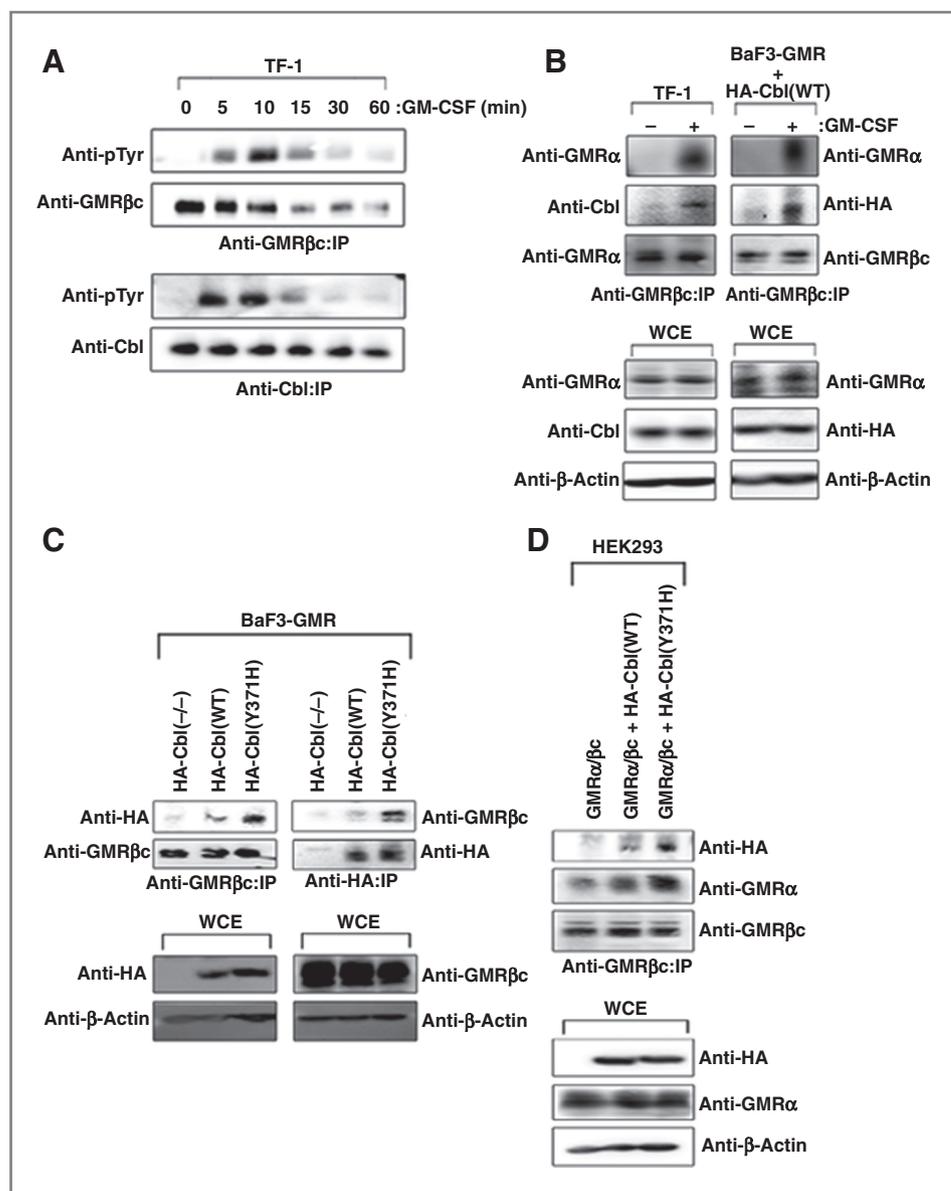
Results

GMR is composed of a ligand-specific α chain (GMR α) and the β common (GMR β) signaling subunit, which is shared with the IL-3 and IL-5 receptors (21). Upon binding of GM-CSF to GMR α , a higher-order signaling complex is formed that promotes the activation of nonreceptor tyrosine kinases JAK2 and Src family kinases c-Src and Lyn, which subsequently

phosphorylate GMR β (22). Activated GMR serves as a docking site for adaptors and signaling molecules resulting in activation of downstream signaling, including the ERK pathway (23). However, the role of Cbl in GMR-mediated signaling has been unexplored.

To begin addressing this question, we first asked whether phosphorylation of Cbl is associated with GMR activation. GM-CSF treatment of serum-starved human myeloid TF-1 cells, which are dependent on GM-CSF for growth and survival (24), promoted phosphorylation of endogenous Cbl in a manner temporally similar to the phosphorylation pattern of GMR β (Fig. 1A and Supplementary Fig. S1). Importantly, endogenous Cbl coprecipitated with endogenous GMR β and GMR α in the presence of GM-CSF in TF-1 cells (Fig. 1B and data not shown). In addition, murine pro-B BaF3 cells, which were previously transduced with shRNA against endogenous murine Cbl (3),

Figure 1. Cbl(Y371H) exhibits increased binding to GMR β . **A**, serum/cytokine starved TF-1 cells for 18 hours were treated with or without 1 ng/mL of GM-CSF for indicated times, then lysed, and immunoprecipitated with anti-GMR β or anti-Cbl antibody and immunoblotted with anti-pTyr antibody. **B**, TF-1 and BaF3-GMR-HA-Cbl(WT) cells were serum/cytokine starved for 18 hours and then treated with 1 ng/mL of GM-CSF for 10 minutes. Equal amount of cell lysates were immunoprecipitated with anti-GMR β antibody and immunoblotted with indicated antibodies. **C**, BaF3-GMR-HA-Cbl (null, WT, or Y371H) cells were lysed, immunoprecipitated, and immunoblotted with the indicated antibodies. **D**, HEK293 cells transfected with the indicated plasmids (right) were lysed, immunoprecipitated, and immunoblotted with the indicated antibodies. WCE, whole cell extract; IP, immunoprecipitation.



were stably reconstituted with ectopic GMR and endogenous level of HA-tagged human Cbl(WT or Y371H). Consistent with TF-1 results, ectopic GMR β c associated with HA-Cbl(WT) and, as expected, GMR α in the presence of GM-CSF (Fig. 1B). These results show for the first time that Cbl is in a complex with GMR preferentially upon GM-CSF stimulation.

In comparison to Cbl(WT), the JMML-associated Cbl (Y371H) mutant showed markedly increased binding to GMR β c in both BaF3-GMR cells and human embryonic kidney epithelial HEK293 cells ectopically expressing GMR α / β c in combination with HA-Cbl(WT or Y371H; Fig. 1C and D), which suggest that Cbl(Y371H) mutant binds to GMR β c with greater

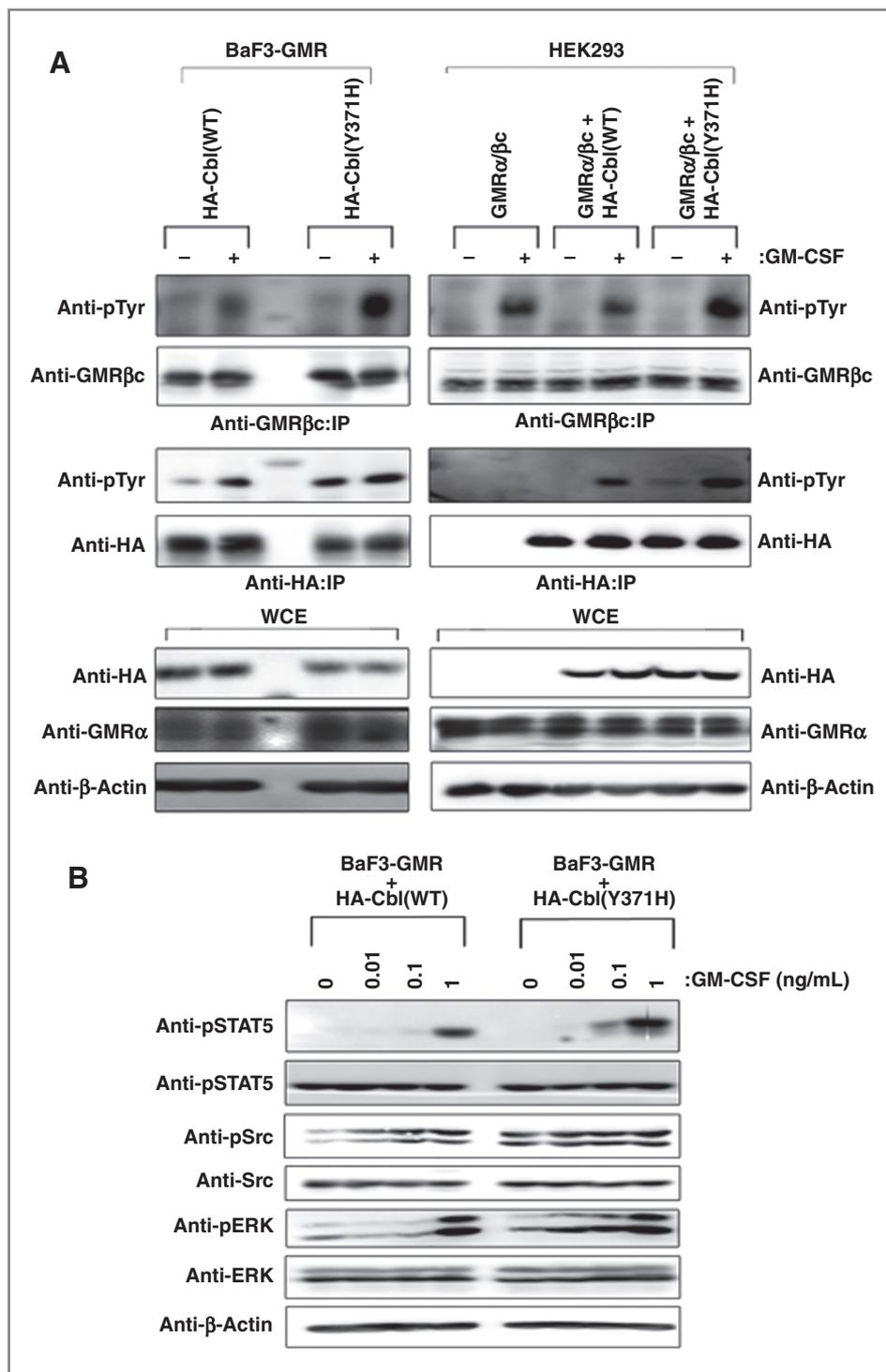


Figure 2. Cbl(Y371H) and the associated GMR β c show hyperphosphorylation profile following GM-CSF stimulation. **A**, BaF3-GMR-HA-Cbl(WT or Y371H) cells (left) or HEK293 cells transfected with the indicated plasmids (right) were serum/cytokine starved for 18 hours and treated with (+) or without (-) 1 ng/mL of GM-CSF for 10 minutes. The cells were lysed, immunoprecipitated with anti-GMR β c or anti-HA antibody, and immunoblotted with anti-pTyr antibody. **B**, serum/cytokine-starved BaF3-GMR-HA-Cbl(WT or Y371H) cells treated for 10 minutes with indicated concentrations of GM-CSF were lysed and immunoblotted with the indicated antibodies. WCE, whole cell extract; IP, immunoprecipitation.

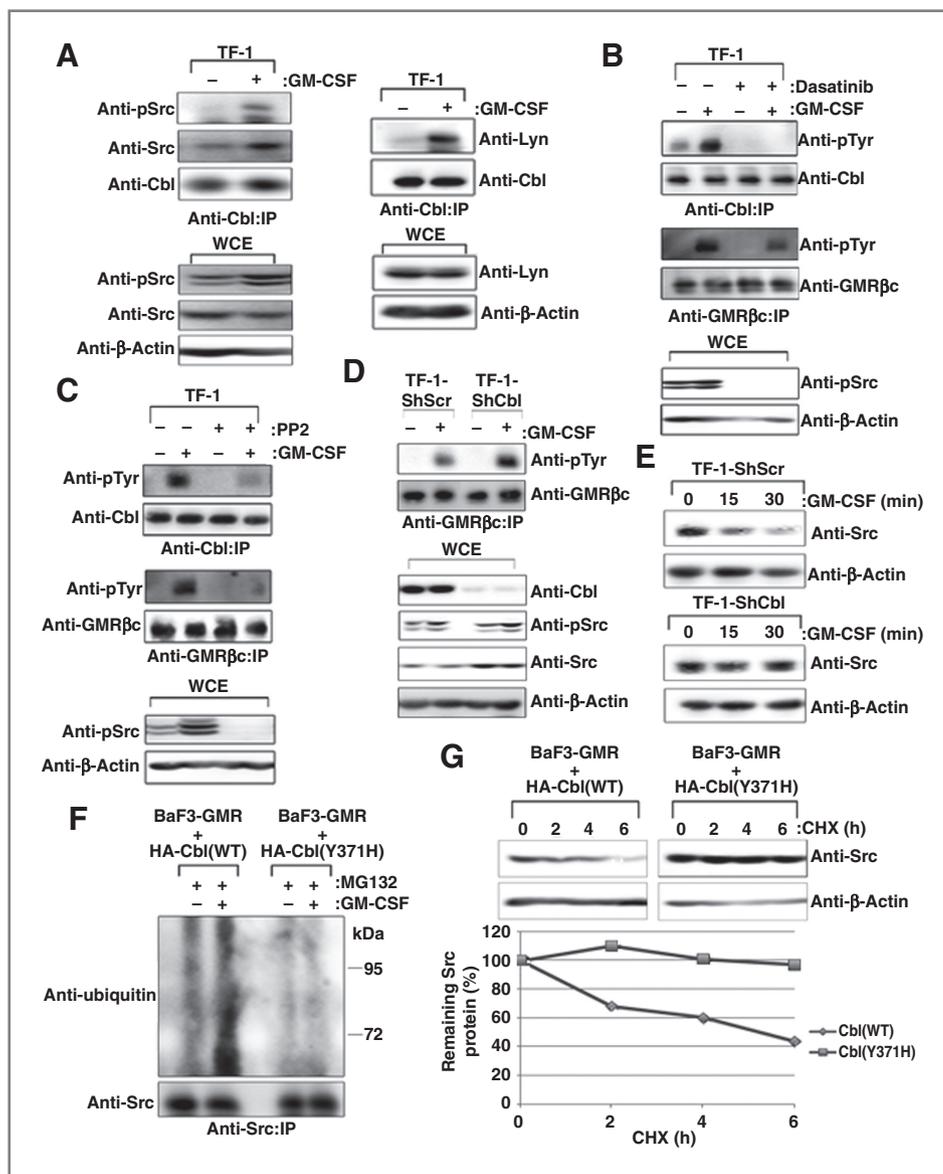


Figure 3. Cbl mediates the ubiquitylation of Src following GM-CSF stimulation. **A**, serum/cytokine starved TF-1 cells treated with (+) or without (–) 1 ng/mL of GM-CSF were lysed, immunoprecipitated with anti-Cbl antibody or anti-Lyn antibody, and immunoblotted with the indicated antibodies. **B**, serum/cytokine-starved TF-1 cells pretreated with 100 nmol/L of dasatinib (+) or DMSO (–) for 1 hour were treated with (+) or without (–) 1 ng/mL of GM-CSF. The cells were lysed, immunoprecipitated with anti-Cbl or anti-GMR β c antibody, and immunoblotted with anti-pTyr antibody. **C**, serum/cytokine-starved TF-1 cells pretreated with 10 mmol/L of PP2 (+) or DMSO (–) for 1 hour were treated with (+) or without (–) 1 ng/mL of GM-CSF. The cells were lysed, immunoprecipitated with anti-Cbl or anti-GMR β c antibody, and immunoblotted with anti-pTyr antibody. **D**, TF-1-(shScr or shCbl) cells serum/cytokine starved for 18 hours were treated with (+) or without (–) 1 ng/mL GM-CSF, were lysed, immunoprecipitated with anti-GMR β c antibody, and immunoblotted with anti-pTyr antibody. Equal amounts of corresponding cell lysate were immunoblotted with indicated antibodies. **E**, TF-1-(shScr or shCbl) cells serum/cytokine starved for 18 hours were treated with 1 ng/mL of GM-CSF for indicated time points, were lysed, and immunoblotted with the indicated antibodies. **F**, BaF3-GMR-HA-Cbl(WT or Y371H) cells pretreated for 3 hours with 10 mmol/L of MG132 (+) or DMSO (–) were treated with (+) or without (–) 1 ng/mL of GM-CSF. The cells were lysed, immunoprecipitated with anti-Src antibody, and immunoblotted with anti-ubiquitin antibody. **F**, BaF3-GMR-HA-Cbl(WT or Y371H) cell treated with 100 mg/mL cycloheximide (CHX) for indicated time points were lysed and immunoblotted with the indicated antibodies. The graph depicts results obtained by normalizing total Src level to the β -actin loading control and plotting the obtained densitometry values. IP, immunoprecipitation.

affinity than Cbl(WT). Notably, HA-Cbl(Y371H) phosphorylation level was substantially higher than that of HA-Cbl(WT) even in the absence of GM-CSF stimulation (Fig. 2A). Following GM-CSF stimulation, expression of HA-Cbl(Y371H) promoted GMR β c hyper-phosphorylation (Fig. 2A). These results suggest

that the amino acid Y371H substitution exaggerates mutant Cbl and also GMR β c activation. In an effort to define the mechanism responsible for the hyper-phosphorylation of Cbl (Y371H) and the associated GMR β c, we next examined the signaling events downstream of GMR. Consistent with the

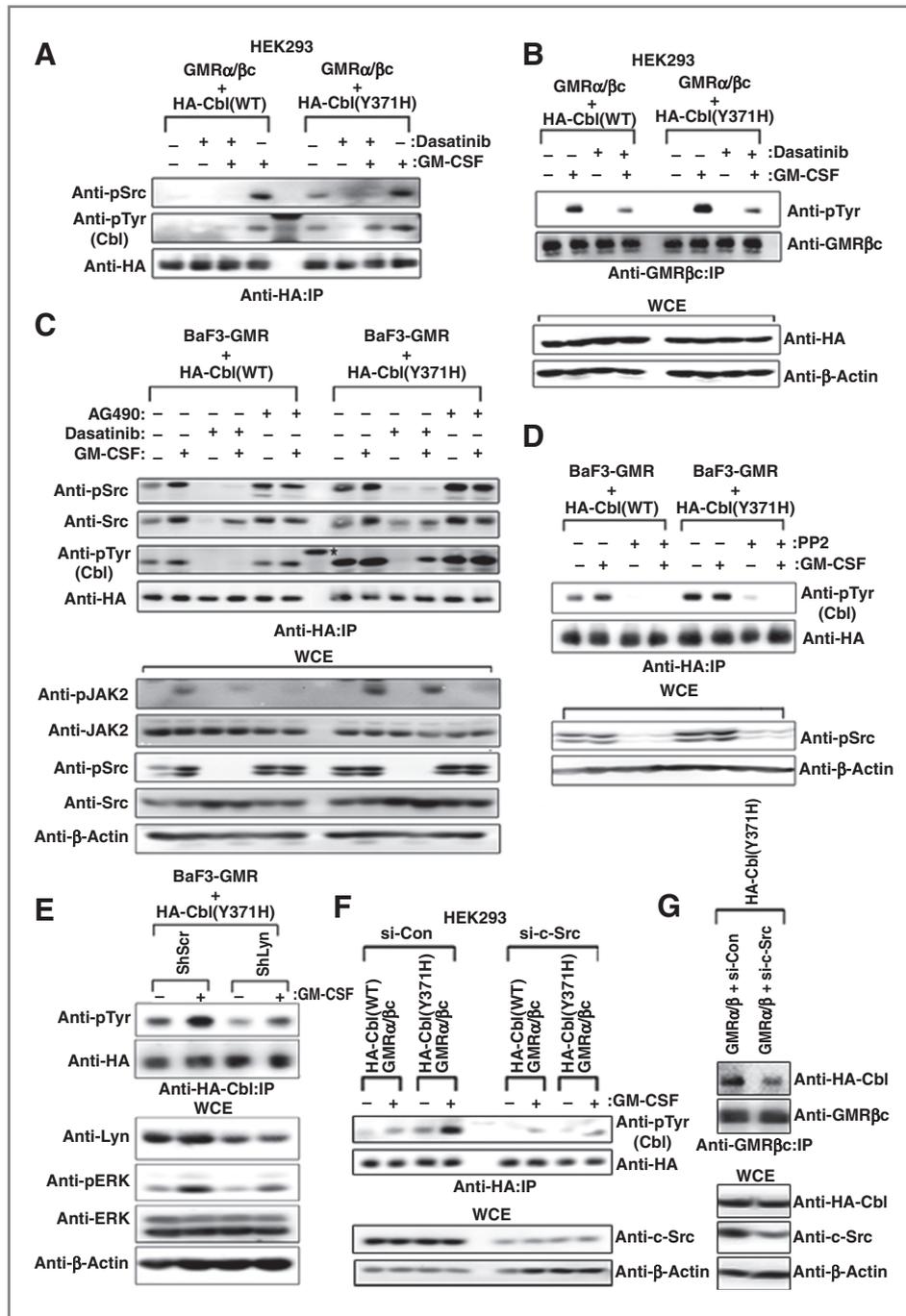
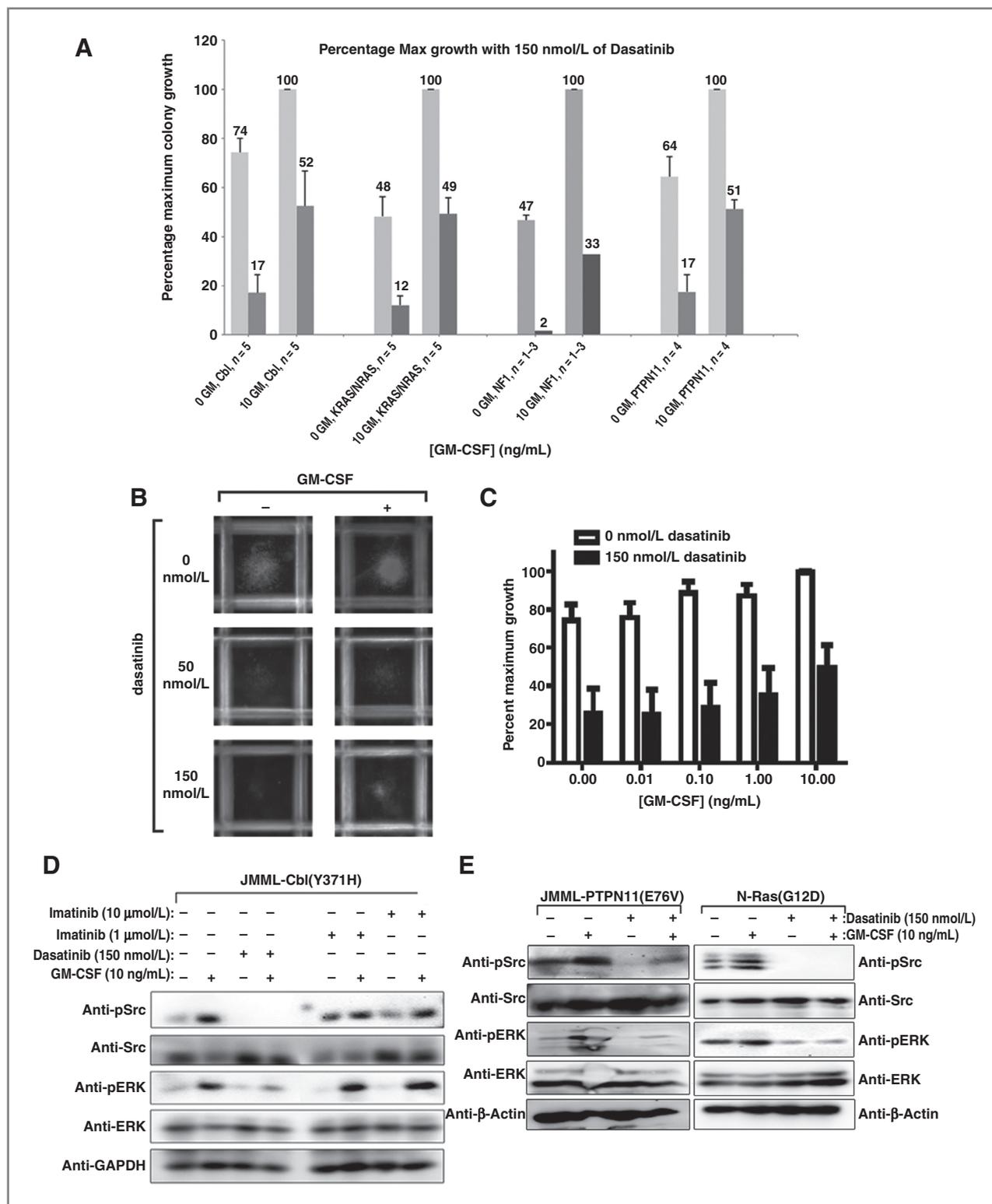


Figure 4. Cbl(Y371H) hyper-phosphorylation is Src-dependent. HEK293 cells transfected with the indicated plasmids pretreated with 100 nmol/L of dasatinib (+) or DMSO (-) for 1 hour and treated with (+) or without (-) 1 ng/mL of GM-CSF for 10 minutes were lysed, immunoprecipitated with anti-HA antibody (A) or anti-GMR β c antibody (B), and immunoblotted with indicated antibodies. C, BaF3-GMR-HA-Cbl(WT or Y371H) cells were serum/cytokine starved for 18 hours and then pretreated with 5 mmol/L of AG490, 100 nmol/L of dasatinib (+), or DMSO (-) for 1 hour and treated for 10 minutes with (+) or without (-) 1 ng/mL of GM-CSF were lysed, immunoprecipitated with anti-HA antibody, and immunoblotted with the indicated antibodies. D, BaF3-GMR-HA-Cbl(WT or Y371H) cells were serum/cytokine starved for 18 hours and then pretreated with 10 mmol/L of PP2 (+) or DMSO (-) for 1 hour and treated for 10 minutes with (+) or without (-) 1 ng/mL of GM-CSF, lysed, immunoprecipitated with anti-HA antibody, and immunoblotted with anti-pTyr antibody. E, BaF3-GMR+ Cbl(Y371H) cells transduced with lenti-shScrambled (shScr) or -shLyn (shLyn) were serum/cytokine starved for 18 hours and treated with (+) or without (-) 1 ng/mL GM-CSF, lysed, immunoprecipitated with anti-HA-Cbl antibody, and immunoblotted with anti-pTyr antibody. Equal amounts of corresponding cell lysate were immunoblotted with indicated antibodies. F, HEK293 cells transfected with c-Src-specific siRNA (si-c-Src) or a nontargeting scrambled siRNA (si-Con) in combination with the indicated plasmids were treated with (+) or without (-) 1 ng/mL of GM-CSF for 10 minutes. Cells were then lysed, immunoprecipitated with anti-HA antibody, and immunoblotted with indicated antibodies. G, HEK293 cells transfected with c-Src-specific siRNA (si-c-Src) or a nontargeting scrambled siRNA (si-Con) in combination with the indicated plasmids were lysed, immunoprecipitated with anti-GMR β c antibody, and immunoblotted with indicated antibodies. WCE, whole cell extract; IP, immunoprecipitation.



hypersensitive GM-CSF phenotype previously reported for JMML progenitors with the Cbl(Y371H) mutation (3), BaF3-GMR-Cbl(Y371H) cells displayed elevated STAT5 and ERK phosphorylation upon exposure to a range of GM-CSF concentrations compared to BaF3-GMR-Cbl(WT) cells (Fig. 2B). Perhaps most importantly, the basal as well as GM-CSF-stimulated Src phosphorylation on Y416, representing activated Src (25), were markedly increased in BaF3-GMR-Cbl(Y371H) cells (Fig. 2B).

Yokuchi and colleagues (26) previously showed that Src phosphorylates Cbl, which triggers its E3 ligase function, including ubiquitin-mediated degradation of Src as a negative feedback. We previously showed that the loss of E3 activity prolonged the turnover rate of Cbl(Y371H) because of a failure in ubiquitin-mediated autodestruction (3). Therefore, we asked whether the loss of E3 activity via the acquisition of Y371H mutation results in the loss of negative regulation of Src and the perpetuation of Cbl(Y371H) and Src activation in the context of GMR signaling. GM-CSF treatment of serum/cytokine-starved TF-1 cells enhanced Src phosphorylation and its interaction to endogenous Cbl (Fig. 3A, left). In particular, Lyn, the predominant Src kinase expressed in myeloid cells (27) shown previously to mediate GMP β c receptor activation and subsequent signaling (22), bound Cbl preferentially in the presence of GM-CSF (Fig. 3A, right). These results show for the first time that Cbl associates with Src, in particular Lyn, in the context of GMR activation in myeloid cells.

Inhibition of Src activity via a specific Src kinase inhibitor, PP2, as well as an U.S. Food and Drug Administration (FDA)-approved Abl and Src tyrosine kinase inhibitor, dasatinib, abolished Src phosphorylation, and dramatically reduced GM-CSF-induced Cbl and GMR β c phosphorylation (Fig. 3B and C), which support the notion that Src is critical in regulating the activity of Cbl and GMR following GM-CSF stimulation. Moreover, endogenous Cbl knockdown in TF-1 cells using *CBL*-specific lentivirus-shRNA, but not the non-targeting scrambled lentivirus-shRNA, enhanced Src and GMR β c phosphorylation, and prolonged Src turnover upon GM-CSF stimulation (Fig. 3D and E). Furthermore, Cbl(WT), but not Cbl(Y371H), promoted GM-CSF-induced Src ubiquitylation and clearance (Fig. 3F and G). These results suggest that JMML-associated Y371H mutation abrogates Cbl-mediated ubiquitylation and subsequent degradation of Src upon GMR activation. In addition, pharmacologic inhibition of Src using dasatinib or PP2 dramatically attenuated hyper-phosphorylation of Cbl(Y371H) and GMR β c upon GM-CSF stimulation (Fig. 4A–D). The heightened phosphorylation of Cbl(Y371H) in the absence of ligand was likewise abrogated in the presence of dasatinib or PP2 (Fig. 4A, C, and

D). Consistent with these observations, shRNA-mediated knockdown of Lyn in BaF3-GMR cells markedly attenuated Cbl(Y371H) phosphorylation, as well as the level of phosphorylated ERK, in the presence or absence of GM-CSF (Fig. 4E). Notably, siRNA-mediated knockdown of c-Src in HEK293 cells not only suppressed Cbl(Y371H) phosphorylation, but also attenuated the association between Cbl(Y371H) and GMR β c (Fig. 4F and G). It should be noted that a highly specific JAK2 inhibitor, AG490, effectively blocked JAK2 phosphorylation, but did not attenuate Src phosphorylation and failed to inhibit Cbl(Y371H) hyper-phosphorylation upon GM-CSF stimulation (Fig. 4C). These results show that the binding of Cbl to GMR β c as well as phosphorylation of Cbl upon GMR activation is dependent on Src, and that JMML-associated Cbl(Y371H) prolongs hyperactivation of GMR-mediated signaling because of a failure in the Cbl-mediated negative regulation of Src.

We next exposed freshly isolated mononuclear cells from peripheral blood and bone marrow collected from multiple JMML patients with confirmed *CBL* mutation (or canonical Ras pathway alteration, including *NF1*, *NRAS*, and *PTPN11* mutations) to increasing doses of dasatinib in colony-forming unit-granulocyte macrophage (CFU-GM) assay (Fig. 5A and Supplementary Table S1). Notably, dasatinib is a multi-kinase inhibitor that targets Src family kinases, BCR-ABL, PDGFRA, and KIT, among others, and is an FDA approved agent commonly used in the treatment of patients with BCR/ABL positive chronic myelogenous leukemia (CML) or in combination with chemotherapy in Ph+ acute lymphoblastic leukemia. Importantly, it is a well-tolerated oral agent with an established toxicity profile published not only in adults but also in children (28, 29) and can be administered as a liquid suspension to young patients incapable of swallowing pills. Strikingly, all tested JMML cells irrespective of mutational status showed substantial inhibition of spontaneous growth in the presence of dasatinib concentrations as low as 10 nmol/L, whereas approximately 50% inhibition at doses of 50 nmol/L or higher was observed in the presence of saturating GM-CSF (Fig. 5A and Supplementary Fig. S2A and S3A). In addition to the diminution in colony number, the size of the colonies was also dramatically reduced in the presence of dasatinib with or without GM-CSF irrespective of mutational status (Fig. 5B and Supplementary Fig. S2B). Exposing cells to dasatinib at a concentration achievable in patients taking the oral drug (150 nmol/L) shifted the GM-CSF hypersensitivity curve back to a more normal appearance (Fig. 5C). In primary peripheral blood mononuclear cells harboring Cbl(Y371H), Cbl intron 8 deletion, PTPN11 (E76V), N-Ras(G12D), or NF1 LOH, dasatinib treatment, which predictably attenuated phosphorylated (p)Src levels,

C, JMML patient-derived leukemia cells ($n = 3$, all with *CBL* mutations) were plated in a CFU-GM hypersensitivity assay with and without dasatinib (150 nmol/L). Percentage of maximum growth at 10 μ mol/L GM-CSF is displayed with and without dasatinib. Error bars represent SEM for these 3 samples. A paired *t* test comparing percentage of maximum growth with and without dasatinib at all concentrations was statistically significant (2-tailed $P \leq 0.0001$). D, primary peripheral mononuclear cells collected from a patient with a Cbl(Y371H) mutation were stimulated with or without GM-CSF in the presence or absence of dasatinib or imatinib. Cell lysates were then prepared and immunoblotted with the indicated antibodies. E, primary peripheral mononuclear cells collected from a JMML patient with a PTPN11(E76V) mutation or with a N-Ras(G12D) mutation were stimulated with or without GM-CSF in the presence or absence of dasatinib. Cell lysates were then prepared and immunoblotted with the indicated antibodies.

dramatically reduced pERK levels in the presence of saturating doses of GM-CSF (Fig. 5D and E and Supplementary Fig. S4). In contrast, imatinib, an Abl-specific inhibitor that does not target Src, had no discernable effect on pSrc or pERK levels or on the hypersensitive colony growth (Fig. 5D and Supplementary Fig. S3B). These results suggest that pharmacologic inhibition of Src attenuates GM-CSF hypersensitivity of peripheral blood and bone marrow-derived primary JMML cells in a dosage-dependent manner.

Discussion

Recently, we and others identified sporadic and germline *CBL* mutations in JMML patients with Y371H mutation emerging as the most common *CBL* mutation (3, 7). Although mutations in Cbl were predicted to result in the loss of E3 function (3, 7), the molecular mechanism by which these mutations cause JMML and more precisely the observed GM-CSF hypersensitivity that underlies JMML remained unclear. Here, we show that Cbl binds to ligand-engaged GMR in a Src-dependent manner and targets Src for ubiquitin-mediated destruction. In comparison to wild-type Cbl, Cbl(Y371H) exhibited increased affinity for activated GMR complex and failed to ubiquitylate active Src, thereby causing accumulation of pSrc. Conformational change of Cbl structure because of gene mutation likely altered the binding affinity of Cbl(Y371H) to GMR β c. The interaction between Cbl and GMR β c is, however, also influenced by GMR activation in the presence of GM-CSF, which raises the possibility that the consequential phosphorylation of Cbl and/or GMR β c promotes their physical association. Notably, Cbl and GMR β c association and phosphorylation in the presence of GM-CSF are, at least in part, dependent on Src. These observations suggest that in addition to gene mutation-induced conformational changes, Src-dependent phosphorylation contributes to Cbl binding to GMR β c. Src has been shown to activate and phosphorylate Cbl on multiple tyrosine residues, including Y700, 731, and 774, which promote several signaling cascades (30). Src also phosphorylates Cbl on Y371 to activate the E3 ligase function of Cbl, which in turn trigger interdependent ubiquitylation and degradation of both proteins (26), a process necessary for terminating downstream signaling postactivation. Consistent with this notion, the pathological stabilization of pSrc resulted in Src-dependent hyper-phosphorylation and concomitant stabilization of Cbl(Y371H) upon GM-CSF stimulation to accentuate and prolong GMR β c phosphorylation and downstream signaling. These lines of evidence suggest that the critical loss of negative regulation of Src upon JMML-causing *CBL* mutations underlies GM-CSF hypersensitivity.

Despite the fact that IL-3 α and IL-5 α also associate with β common subunit (i.e., GMR β c; ref. 21), JMML cells do not display hypersensitivity to IL-3 or IL-5. Notably, Src binds to the ligand-specifying GMR α chain, which upon ligand binding associates with GMR β c to initiate GMR β c activation via JAK2 and Src (22, 31). However, it is unclear whether IL-3R or IL-5R directly interacts Src or Cbl and whether the interplay observed between Cbl and Src on GMR is recapitulated on IL-3/5R. For

example, IL-3 treatment was previously shown to increase the amount of Grb2 associated with Cbl; however, it did not alter the level of Fyn (another member of Src kinase family) associated with Cbl (32). Consistent with these uncertainties, IL-3 treatment did not differentially influence IL-3-mediated signaling in BaF3-Cbl(Y371H) cells in comparison to wild-type Cbl-expressing BaF3 cells (data not shown). These observations underscore the importance of Src interaction on GMR α in promoting GM-CSF hypersensitivity observed in cells harboring Cbl(Y371H) mutation.

Perhaps most importantly, dasatinib dramatically attenuated spontaneous and GM-CSF-dependent hypersensitive growth phenotype of human primary leukemic cells collected from peripheral blood or bone marrow of JMML children harboring Cbl mutations. Notably, imatinib, an Abl-specific inhibitor that does not target Src, had no appreciable effect on pSrc or pERK levels or on the hypersensitive colony growth of JMML cells. Intriguingly, dasatinib also reduced the hypersensitive growth phenotype of JMML cells harboring Ras pathway mutations (see Fig. 5A, E, Supplementary Fig. 2 and 4), which suggests potential involvement of Src in non-Cbl mutated JMML cells. Although the connection between Ras pathway and Src in the context of GMR-mediated signaling has not been formally established, a biochemical link between Src and Ras has been speculated through the interaction via Raf-1(33). Recently, the expression of oncogenic Ras has been shown to induce c-Src activation, and elevated Src activity has been observed in human cancer cell lines that harbor oncogenic Ras mutations (34) as well as in pancreatic ductal adenocarcinoma that are characterized with a high incidence of oncogenic *K-Ras* mutations (35). Interestingly, G proteins are GTPases that have also been shown to directly activate Src (36). Thus, it remains to be determined whether oncogenic Ras, which is also a GTPase, drives Src activity via Raf-1 or yet-defined mediator protein(s) to influence Cbl-dependent GMR signaling. Undefined connection between Ras pathway and Src notwithstanding, these results suggest that the loss of negative regulation of Src in certain susceptible myeloid progenitor population is central to the GM-CSF hypersensitivity phenotype invariably observed in JMML patients, and that Src represents a potential druggable target for the amelioration of the hypersensitive growth phenotype of primary human JMML cells. Despite the promise of dasatinib, it is a multi-kinase inhibitor, and hence the future challenge would be to develop small molecule inhibitors that exclusively target Src ideally in leukemic progenitors in JMML patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Bunda, M.S. Irwin, M.L. Loh, M. Ohh

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Bunda, M.W. Kang, S.S. Sybingco, J. Weng, H. Favre, M.L. Loh

Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): S. Bunda, M.W. Kang, S.S. Sybingco, J. Weng, H. Favre, M.S. Irwin, M.L. Loh, M. Ohh

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