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# Role of BCR-ABL-Y177 mediated p27kip1 phosphorylation and cytoplasmic localization in enhanced proliferation of chronic myeloid leukemia progenitors

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## Abstract

In chronic myelogenous leukemia (CML) hematopoietic stem cell transformation leads to increased proliferation of malignant myeloid progenitors. The cyclin-dependent kinase inhibitor p27kip1 (p27) is a critical negative regulator of hematopoietic progenitor proliferation and pool size that is deregulated in BCR-ABL expressing cell lines. However, cell-context specific regulation of p27 in primary human CML progenitors and its contribution to CML progenitor expansion remain unclear. Here we investigated p27 regulation and function in (1) CD34+ cells from CML patients and (2) human CD34+ cells ectopically expressing the BCR-ABL gene following retrovirus transduction. We found that p27 levels are increased in CML CD34+ cells related to a BCR-ABL dependent increase in p27 protein translation. However p27 was relocated to the cytoplasm in CML progenitors and nuclear p27 levels were reduced, allowing increased cell cycling and expansion in culture. Cytoplasmic relocation of p27 in CML progenitors was related to signaling through BCR-ABL Y177, activation of the AKT kinase and phosphorylation of p27 on Thr-157 (T157). Expression of a mutant p27 that cannot be phosphorylated on T157 significant inhibited CML progenitor proliferation. These studies demonstrate the importance of BCR-ABL-Y177-AKT mediated p27 phosphorylation in altered p27 localization and enhanced proliferation and expansion of primary CML progenitors.

### Keywords

Chronic myeloid leukemia; Hematopoietic stem and progenitor cells; BCR-ABL; Leukemia stem cells



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**Author contributions.** SC: designed and performed research, analyzed data, wrote manuscript; TM: performed experiments, reviewed manuscript; RB: designed study, analyzed data, wrote manuscript

## Introduction

Chronic myeloid leukemia (CML) is a hematopoietic stem cell (HSC) malignancy characterized by the BCR-ABL gene rearrangement.(1) CML is sustained by a rare population of BCR-ABL+ HSC with multi-lineage capacity that generate a expanded pool of malignant myeloid progenitors.(2) The BCR-ABL protein has constitutive, enhanced tyrosine kinase activity resulting in the deregulation of a number of growth regulatory pathways.(1) CML progenitors demonstrate enhanced proliferation related to increased cell cycle progression through the G1-S phase.(3–5) The proliferative effects of BCR-ABL require a tyrosine 177 (Y177) motif in the BCR portion of the protein. Tyrosine phosphorylated BCR-ABL Y177 interacts with the Grb2 adapter protein SH2 domain leading to assembly of signaling complexes that mediate activation of critical downstream pathways.(6, 7) Mutation of Y177 (Y177F) leads to markedly reduced proliferation of BCR-ABL expressing murine and human myeloid progenitor cells.(8–10)

The cyclin-dependent kinase inhibitor p27kip1 (p27; cyclin-dependent kinase inhibitor 1B) is an important cell cycle negative regulator of mammalian cells. Nuclear p27 functions to inhibit the activity of cyclin/cdk complexes during G0 and G1.(11, 12) p27 expression is regulated mainly at the posttranscriptional level but is also subject to transcriptional regulation.(13) Posttranscriptional regulation of p27 can occur via modulation of protein translation,(14) or distribution between the nucleus and cytoplasm. AKT mediated phosphorylation at Thr-157 (T157) of p27 promotes cytoplasmic localization and inhibits nuclear import.(15–17) p27 is also regulated via 26S proteasomal degradation, following phosphorylation of Thr-187 (T187) residue of p27 by cyclin E-Cdk2 complex, transport from the nucleus to the cytoplasm by the p27/CSN5 and the COP9 signalosome complex, and ubiquitination by the SCF Skp2 complex.(18, 19) Although the p27 gene is rarely mutated in human tumors, p27 protein expression is frequently downregulated through a variety of mechanisms, and a tumor suppressing function of p27 is reported.(20, 21) Several oncogenic tyrosine kinases can impair p27 function and expression. In AML cells, mutant Flt3 kinase can inhibit Foxo-mediated p27 expression.(22) In breast cancer Src activation downstream of EGFR and Her2 receptor tyrosine kinases can impair p27 activity and stability.(23, 24)

Studies with knockout mice show that p27 plays an important role in regulating hematopoietic progenitor proliferation and pool size.(25) Several defects in p27 regulation have been described in BCR-ABL expressing cell lines, including reduced FoxO-regulated p27 transcription, enhanced Jab1 mediated export and enhanced Skp2 mediated p27 degradation.(26–29) In contrast to BCR-ABL-expressing immortalized cells lines, p27 levels are reported to be increased in CML CD34+ cells.(30, 31) The cell-context specific regulation of p27 protein in primary CML progenitor cells and the functional importance of p27 alterations in abnormal proliferation of primary CML progenitors are not clear. The aims of the current studies were to investigate p27 regulation and function in CD34+ cells from CML patients and in a human CML cell model based on retrovirus-mediated expression of BCR-ABL in cord blood CD34+ cells. Our results indicate that p27 levels are increased in CML CD34+ cells through BCR-ABL-dependent post-transcriptional mechanisms. However p27 protein is primarily localized in the cytoplasm and nuclear levels

are reduced, resulting in impaired cell cycle regulation. Cytoplasmic translocation of p27 in CML progenitor cells is related to a BCR-ABL Y177 induced AKT-mediated phosphorylation of p27 on T157. Restoration of nuclear localization of p27 significantly inhibits CML progenitor proliferation. These studies provide clear evidence for the importance of BCR-ABL-Y177-AKT mediated p27 phosphorylation and mislocalization in CML pathogenesis.

## Materials and Methods

### Patient samples

Human cord blood (CB) samples and bone marrow (BM) samples from healthy individuals, and BM or peripheral blood samples from CML patients were obtained under protocols approved by the Institutional Review Board at City of Hope, in accordance with assurances filed with and approved by the Department of Health and Human Services, and meeting all requirements of the Declaration of Helsinki. CML patients studied were in chronic phase and had not received treatment other than Hydroxyurea. Mononuclear cells were isolated using Ficol separation. CD34+ cells were selected using the StemSep CD34 isolation kit (Stem Cell Technologies Inc., Vancouver, BC).

### Antibodies and reagents

Antibodies were used for Western blotting and immunofluorescence included: CDKN1B/p27, JAB1, SKP2, tubulin, Sp1 (Santa Cruz Biotechnology Santa Cruz, CA); phospho-pRB (S780) and phospho-pRB (S807/S811) (Cell Signaling Technology, Inc., Danvers, MA); phospho-p27 (T157) and phospho-p27 (T187)(R&D Systems, Minneapolis, MN); anti-Actin (Sigma), C-Abl (Oncogene), HRP-conjugated goat-anti-mouse IgG and HRP-conjugated goat-anti-rabbit IgG (Jackson ImmunoResearch). LY294002 was obtained from Sigma-Aldrich; St. Louis, MO and Imatinib from Novartis Pharmaceuticals.

### Retroviral vectors

MIG R1, MIG 210 and MIG 210-Y177F have been previously described (8) MIG R1 expresses the enhanced green fluorescent protein (GFP) gene. MIG 210 and MIG 210-Y177F have p210BCR-ABL and P210BCR-ABLY177F genes inserted upstream of the IRES upstream of GFP. EYFP-p27 constructs were released from pEYFP-C1 p27 and pEYFP-C1 T157A vectors expressing the human p27 and p27 T157A genes by digestion with Nhe1 and Sal1.(15) The IRES and GFP fragment were removed from the MIG-R1 vector by digestion with Hap1 and Sal1, and the YFP-p27 constructs were inserted after blunting of the Nhe1 site generating the MEYFP p27 and MEYFP p27T157A vectors.

### Retroviral transduction of CD34+ cells

Infectious virus particles were produced and CD34+ cell transduction performed as previously described.(32) Subsequently cells were labeled with anti-CD34-APC antibodies (Becton Dickinson, San Jose, CA) and $CD34^{+}GFP^{+}$ or $CD34^{+}YFP^{+}$ cells collected by flow cytometry sorting (MoFlo, Cytomation Inc., Fort Collins, CO).

### Cell culture

$CD34^+GFP^+$ or $CD34^+YFP^+$ cells were cultured in serum free medium (SFM) supplemented with low concentrations of GF at concentrations similar to that found in stroma-conditioned medium from long-term bone marrow cultures.(33) Cell expansion was measured by counting the number of cells generated after culture for specified number of days.

### Western Blotting

Western blotting was performed as previously described.(34) AKT kinase activity was measured using a kit from Cell Signaling Technology. AKT was immunoprecipitated and *in vitro* kinase reactions performed using glycogen syntheses kinase–3 (GSK-3) as substrate. Reaction products were subjected to Western blotting with antibodies to phospho–GSK-3α/β. One third of the lysate was retained for Western blotting for actin to check loading. For nuclear-cytoplasmic fractionation cells were lysed in hypotonic buffer for 5 min, gently pipetted for 1 min on ice and centrifuged at 13,000 rpm, 4°C for 10 s. Supernatants were collected as the cytoplasmic extract. After washing with hypotonic buffer, nuclear pellets were incubated in high-salt buffer at 4°C for 30 min, and supernatants collected as nuclear extracts after centrifugation at 13,000 rpm, 4°C for 5 min.(35)

### Metabolic labeling of p27 protein

BCR-ABL and control GFP vector transduced CD34+ cells were cultured for 11 days to obtain sufficient numbers of cells for study. Cells were starved in methionine/cysteine free DMEM medium supplemented with 5% dialyzed FBS (Invitrogen) for 90min. Cells were labeled with 250μCi /ml [$S^{35}$] methionine/cysteine mixture (PerkinElmer) for 90 minutes, suspended in isotope free DMEM with 10% FBS and excess methionine and cysteine (0.1mg/ml) and analyzed either immediately (0 hours) or after 1 hour of incubation. 1.5 mg protein extract was cleared using Protein A beads (Pierce Chemical Company) at 4°C for 1 hour, incubated with anti-p27 antibody overnight at 4°C (2μg) (Santa Cruz), and incubated with 30μl True Blot beads (eBioscience) for 2 hours. Beads were isolated by centrifugation, washed and boiled with 2x sample loading buffer, resolved by SDS-PAGE, visualized using autoradiography and quantified using densitometry.

### Immunofluorescence staining

Cells ($3\times10^3$) were deposited on glass slides by cytocentrifugation, fixed in cold 4% paraformaldehyde and permeabilized in PBS containing 0.3% BSA, 0.5% Triton X-100. Slides were blocked using antibody dilution buffer (3% BSA, 0.1% Tween20/PBS) for 30 minutes, incubated with anti-p27 (Santa Cruz) or anti-YFP antibody at room temperature for 2 hours, washed in PBS and with anti-mouse IgG-Texas Red (Jackson) for 1 hour. Following additional washes, coverslips were mounted on glass slides in Anti-fade containing DAPI (Invitrogen). Images were obtained using a Zeiss AxioImager microscope and Zeiss Upright LSM310 Laser Scanning Confocal Microscope.

### Real-time quantitative RT-PCR analysis

RNA was extracted from CD34+ cells using Trizol (Invitrogen/Life Technologies, Carlsbad, CA) and quantitative RT-PCR analysis for detection of p27 transcripts was performed using

a TaqMan real-time one step RT kit and the ABI Prism 7900 sequence detector (Applied Biosystems, Foster City, CA). Hybridization probes and p27 specific primers were purchased from Applied Biosystems (Foster City, CA). β2-microglobulin (β2M) levels were measured as internal controls. p27 and β2M levels were calculated from standard curves.

### Cell Cycle Analysis

CD34+ cells were fixed with 70% ethanol on ice overnight, washed with PBS to remove residual ethanol and resuspended in cell cycle buffer [PBS, RNAse A (0.1mg/ml), Propidium iodide (100μg/ml)] at a concentration of $10^6$cells/ml, incubated at room temperature for 30 minutes and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and ModFit software (Verity Software House Inc. Topsham, ME).

## Results

### Increased p27 protein expression in CML CD34+ cells and BCR-ABL expressing cord blood CD34+ cells related to increased protein translation

p27 protein expression was significantly increased in primary CML CD34+ cells compared with normal CD34+ cells on Western blotting (Figure 1A). However p27 protein levels in CML CD34+ cells were reduced after in vitro exposure to Imatinib mesylate suggesting that increased p27 levels are related to BCR-ABL kinase activity (Figure 1B). In addition p27 expression was increased in BCR-ABL transduced cord blood CD34+ cells compared with cells transduced with control vectors expressing GFP alone, further indicating that increased p27 levels are related to BCR-ABL expression (Figure 1C). p27 mRNA levels were similar in BCR-ABL and control vector transduced CD34+ cells on Q-RT-PCR analysis (Figure 1D), suggesting that elevated level of p27 expression in BCR-ABL expressing CD34+cells was likely regulated at the posttranscriptional rather than transcriptional level. Metabolic labeling with $S^{35}$-methionine indicated increased de novo p27 synthesis in BCR-ABL expressing CD34+ cells compared with controls (Figure 1E). Degradation of newly synthesized p27 also appeared to be increased. Western blotting also revealed increased phosphorylation of p27 on T187, increased expression of Jab1, and increased expression of SKP2, consistent with increased p27 degradation through the proteosomal degradation pathway (Figure 1F) These results suggest that increased p27 expression in CML CD34+ cells results from increased protein translation, is directly related to BCR-ABL expression, and occurs despite increased protein degradation.

### Altered localization of p27 in BCR-ABL expressing CD34+ cells, related to BCR-ABL Y177 signaling

We performed immunofluorescence microscopy to examine the sub-cellular distribution of p27 in CD34+ CML cells and BCR-ABL transduced expressing cells. In contrast to normal CD34+ cells, where p27 was located in the nucleus, p27 was located predominantly in the cytoplasm of CML CD34+ cells, with markedly reduced nuclear localization (Figure 2A). Similarly increased cytoplasmic and reduced nuclear p27 was seen in BCR-ABL transduced CD34+ cells indicating that cytoplasmic location of p27 is directly related to BCR-ABL expression (Figure 2B). Cytoplasmic localization of p27 was also seen in the K562 CML blast crisis cell line (Figure 2C). This was confirmed by Western blotting after nuclear and

cytoplasmic fractionation of K562 cells (Figure 2D). We have shown that mutation of the tyrosine 177 motif in BCR-ABL to phenylalanine (Y177F) results in markedly reduced proliferation of BCR-ABL expressing CD34+ cells.(8) Here we observed that expression of BCR-ABL Y177F in cord blood CD34+ cells resulted in reduced p27 expression compared with BCR-ABL expressing cells (Figure 2E). p27 mRNA levels measured by Q-RT-PCR analysis were similar in cell expressing the Y177F and wild type BCR-ABL (Figure 1C), but the Y177F mutation reversed abnormal cytoplasmic localization and restored nuclear localization of p27 suggesting a critical role for Y177-mediated signaling in BCR-ABL induced alteration in p27 localization (Figure 2B).

## Abnormal localization of p27 is associated with altered cell cycle regulation in BCR-ABL expressing CD34+ cells

Sequential phosphorylation of pRB by cyclin D-CDK4 and cyclin E-CDK2 in $G_1$ phase inactivates its growth-inhibitory function allowing cell cycle progression.(36) Nuclear p27 functions to inhibit CDK2 and CDK4 activity resulting in hypophosphorylation of pRB and inhibition of cell cycle progression. We observed increased phosphorylation of pRB on S780 and S807/S811 (CDK4 and CDK2 sites respectively) in BCR-ABL transformed cells suggesting that reduced nuclear p27 is associated with increased CDK activity (Figure 3A). In contrast BCR-ABL Y177F expressing cells demonstrated reduced pRB phosphorylation suggesting restoration of p27 regulatory function. Consistent with this reduced G1 and increased S-phase populations were observed in BCR-ABL expressing CD34+ cells compared to controls, and these changes were reversed in BCR-ABL Y177F expressing cells (Figure 3B). Consistent with our previous studies enhanced growth of BCR-ABL expressing CD34+ cells was significantly reduced in cells expressing BCR-ABL Y177F (Figure 3C). These observations support an important role for BCR-ABL Y177 signaling in altered p27 mediated cell cycle regulation and increased cell growth.

## Role of AKT mediated phosphorylation of p27 T157 in abnormal p27 localization and increased proliferation of CML CD34+ cells

We have previously shown an important role for AKT kinase signaling downstream of BCR-ABL Y177.(8) AKT kinase mediated phosphorylation of p27 at T157 results in cytoplasmic localization of p27 protein and increased cell cycle progression in breast cancer cell lines.(15–17) We observed that p27 phosphorylation on T157 was increased in primary CML CD34+ cells (Figure 4A). BCR-ABL expressing cord blood CD34+ cells also demonstrated increased p27 T157 phosphorylation, which was reversed by the Y177F mutation, indicating the requirement for Y177 mediated signals for this modification (Figure 4B). We confirmed that BCR-ABL expression resulted in increased AKT kinase activity in CD34+ cells, which was reversed by the Y177F mutation (Figure 4C). Treatment of BCR-ABL expressing CD34+ cells with the PI-3 kinase inhibitor LY294002 (25μM) for 24 hours resulted in reduced p27 T157 phosphorylation and in increased nuclear and reduced cytoplasmic localization of p27 (Figures 4D and 4E). BCR-ABL expressing CD34+ cells demonstrated also reduced phospho-pRB levels following LY294002 treatment (Figure 4E). CD34+ cells from CML patients also demonstrated reduced p27 T157 phosphorylation and pRB phosphorylation following LY294002 treatment (Figure 5C). These results support a

role for BCR-ABL Y177 and PI-3K/AKT signaling in altered phosphorylation, aberrant cellular localization and reduced activity of p27 in CML progenitors.

We further investigated the role of T157 phosphorylation in abnormal p27 localization and function in CML progenitors by expressing a p27 protein with a mutation of T157 to Alanine (p27-T157A) that is not subject to phosphorylation at this position by AKT.(15) Retrovirus vectors expressing wild type p27-YFP or p27-T157A-YFP fusion proteins were created and used to transduce CML and normal CD34+ cells (Figure 5A). YFP+ CD34 cells were selected by flow cytometry sorting. In normal CD34+ cells p27-YFP and p27 T157A-YFP both localized to the nucleus (not shown). In CML CD34+ cells, p27-YFP was located predominantly in the cytoplasm, whereas p27 T157A-YFP was localized predominantly in the nucleus (Figure 5B). CML CD34+ cells expressing the T157A-YFP mutant showed reduced p27 T157 phosphorylation on Western blotting (Figure 5C). Treatment with LY294002 did not further reduce T157 phosphorylation. In addition p27 T157A expressing CML CD34+ cells showed reduced level of phospho-pRB (Figure 5C), and significantly reduced growth in culture (Figure 5D). p27 T157A expression also resulted in modest reduction in growth of normal CD34+ cells. These results support an important role for T157 phosphorylation in altered localization and function of p27 in CML progenitors.

## Discussion

The present studies have yielded several important insights regarding altered p27 regulation in primary CML progenitor cells: (1) Total p27 protein levels are increased as a result of enhanced translation, despite increased protein degradation. (2) p27 is located primarily in the cytoplasm and nuclear levels are reduced, resulting in reduced p27 cell cycle regulatory activity. (3) Abnormal p27 regulation is related to BCR-ABL Y177 signaling through the AKT kinase and phosphorylation of p27 on T157. (4) Mutation of the AKT phosphorylation site on p27 results in markedly reduced proliferation of CML progenitors. Together these studies demonstrate a critical role for Y177 and AKT in p27 mislocalization and increased proliferation and expansion of hematopoietic progenitors in CML.

p27 protein levels were increased in both primary CML CD34+ cells and BCR-ABL transduced CD34+cells. However p27 mRNA levels were similar in BCR-ABL expressing cells and control CD34+ cells, suggesting that p27 overexpression is mediated by posttranscriptional mechanisms. Consistent with this metabolic labeling studies demonstrated increased p27 translation in BCR-ABL expressing cells. However, p27 was located primarily in the cytoplasm and nuclear levels of p27 were reduced in CML CD34+ cells. Cytoplasmic relocation of p27 was also seen after ectopic expression of BCR-ABL in normal CD34+ cells confirming that this was BCR-ABL dependent. p27 translocation to the cytoplasm was associated with increased phosphorylation of pRB, cell cycling and cell expansion in culture. These observations indicate that p27 cell cycle inhibitory activity is impaired in CML progenitors despite increased total cellular p27 levels, related to cytoplasmic relocalization of the protein.

The BCR-ABL Y177 autophosphorylation site mediates binding of the GRB2 adapter and plays an essential role in BCR-ABL mediated myeloproliferation in the mouse transduction-

transplantation model and primary human CD34+ cells.(6, 8, 17) BCR-ABL Y177 mediated signals play a critical role in PI-3K-AKT activation in human progenitors through SOS mediated activation of Ras and/or through the adaptor GAB2.(6, 7) AKT mediated phosphorylation of p27 on T157 is associated with cytoplasmic localization in breast and other cancers.(15–17) We show here that p27 is phosphorylated on T157 in CML progenitors and BCR-ABL transduced CD34+cells. Mutation of BCR-ABL Y177 resulted in reduced p27 T157 phosphorylation, relocalization of p27 to the nucleus and reduced cycling of BCR-ABL transformed progenitors. Treatment with a PI-3K inhibitor, LY294002, also resulted in reduced p27 T157 phosphorylation, nuclear relocalization of p27 and reduced proliferation of CML CD34+ cells. These results indicate an important role for BCR-ABL Y177 mediated AKT activation in p27 phosphorylation on T157, relocation to the cytoplasm and cell cycle deregulation in CML progenitors.

p27 protein concentrations are high in quiescent cells, and decrease during G1 phase and S phase, reflecting changes in protein translation.(14, 37, 38) Mitogenic growth factor signaling usually mediates a decrease in p27 protein levels. In contrast we observed increased p27 translation in proliferating CML expressing progenitors in our studies, which is BCR-ABL mediated and Y177 dependent. Aberrant protein translation in CML cells mediated by BCR/ABL effects on RNA binding proteins has been observed, and similar mechanisms may act to modulate p27 expression.(39) Posttranscriptional regulation of p27 by miR-221 and miR-222 has also been reported, and a role of altered microRNA regulation in enhanced p27 translation in CML progenitors deserves exploration.(40) BCR-ABL may down-regulate p27 in cell lines by inhibiting transcription, or by enhancing nuclear export of p27, SKP2 expression and proteasome-mediated degradation.(27, 29, 41) Absence of SKP2 expression can attenuate BCR-ABL-induced myeloproliferative disease.(41) These results differ from the enhanced p27 expression observed in this study and may represent cell context dependent differences in p27 regulation between the BCR-ABL expressing cell lines or murine cells used in previous studies and primary human progenitors used in our experiments. Interestingly we did observe increased p27 T187phosphorylation, JAB1 expression and SKP2 expression in our studies suggesting that increased cytoplasmic p27 expression occurs despite enhanced p27 degradation in CML cells.

Reversal of p27 localization to the cytoplasm, pRB phosphorylation and enhanced CML progenitor proliferation by expression of a p27 T157A mutant which resists phosphorylation strongly supports a role for p27 deregulation in increased proliferation of CML progenitors. Studies using genetic mouse models have demonstrated that p27 regulates hematopoietic progenitor proliferation and pool size but not HSC number, cycling, or self-renewal, which instead are predominantly regulated by another CDKI, p21cip1/waf1.(25, 42) p27-deficient HSCs generate enhanced numbers of progenitors and increase progenitor pool size to eventually dominate blood cell production following competitive transplantation.(42) The phenotype of p27 deleted mice bears similarities to abnormalities in hematopoiesis observed in CML patients and in mouse models of disease, where HSC transformation results in a selective expansion of hematopoietic cells at the myeloid progenitor and subsequent stages. Therefore impairment of p27 function may contribute to selective expansion of the myeloid progenitor pool that characterizes CML. Similar mechanisms may be active in the pathogenesis of other hematological malignancies associated with enhanced AKT activity.

In conclusion, our studies demonstrate that reduced nuclear levels of p27 in CML progenitors are related to BCR-ABL Y177 mediated AKT kinase activation and p27 phosphorylation on T157. Interruption of this signaling pathway restores nuclear localization of p27 and markedly reduces cell cycling and growth of CML progenitors, indicating a critical role for abnormal p27 localization to the cytoplasm in enhanced proliferation and expansion of hematopoietic progenitors in CML.

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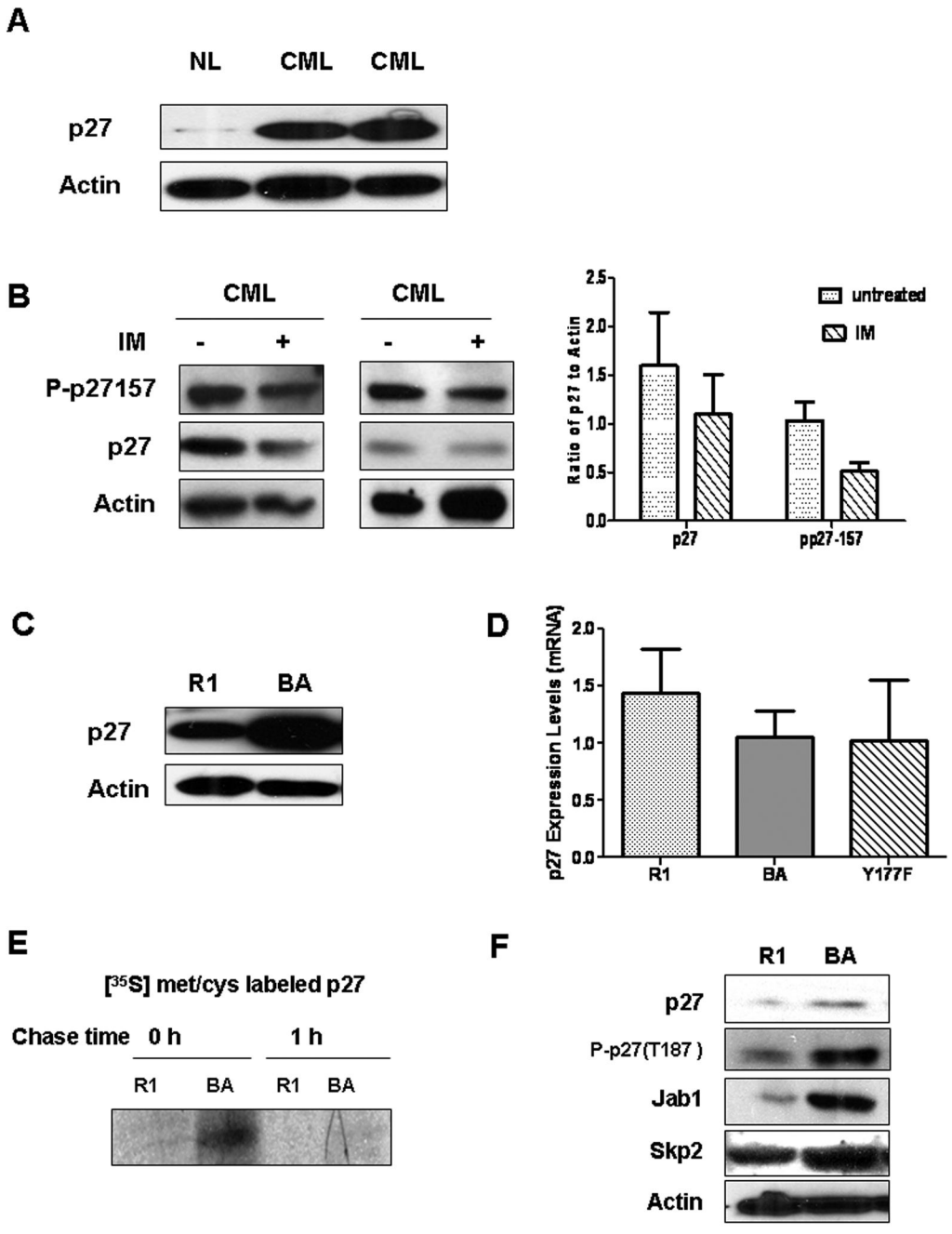


**Figure 1. Enhanced expression of p27 protein in primary CML CD34+ cells and human CD34+ cells ectopically expressing BCR-ABL**

(A) Normal (NL) (BM n=3, CB n=6) and CML (n=6) BM CD34+ cells were analyzed for p27 expression by Western blotting. Blots were labeled with anti-Actin antibodies to check for protein loading. (B) CML CD34+cells (n=4) treated with or without 1uM of Imatinib were analyzed for levels of p27 and T157-phosphorylated p27 by Western blotting. (C) Human CB CD34+ cells (n=5) were transduced with retrovirus vectors expressing GFP alone (R1) or BCR-ABL and GFP (BA). CD34+GFP+ cells were analyzed for p27 expression by Western blotting. (D) p27 mRNA levels in retrovirus-transduced CD34+ cells

(n=7 each) were analyzed using quantitative RT-PCR. The results show the ratio of p27 to β2 microglobulin RNA levels. (E) CD34+GFP+ cells were pulse-labeled with [$S^{35}$] cysteine/methionine and analyzed immediately after labeling or after 1 hour. Protein extracts were prepared and p27 protein immunoprecipitation performed and labeled p27 was detected via autoradiography. (F) Western blot analysis for p27, phospho-p27 T187, JAB1, SKP2 and Actin expression in retrovirus-transduced CD34+ cells expressing GFP alone (R1) or BCR-ABL and GFP (BA).

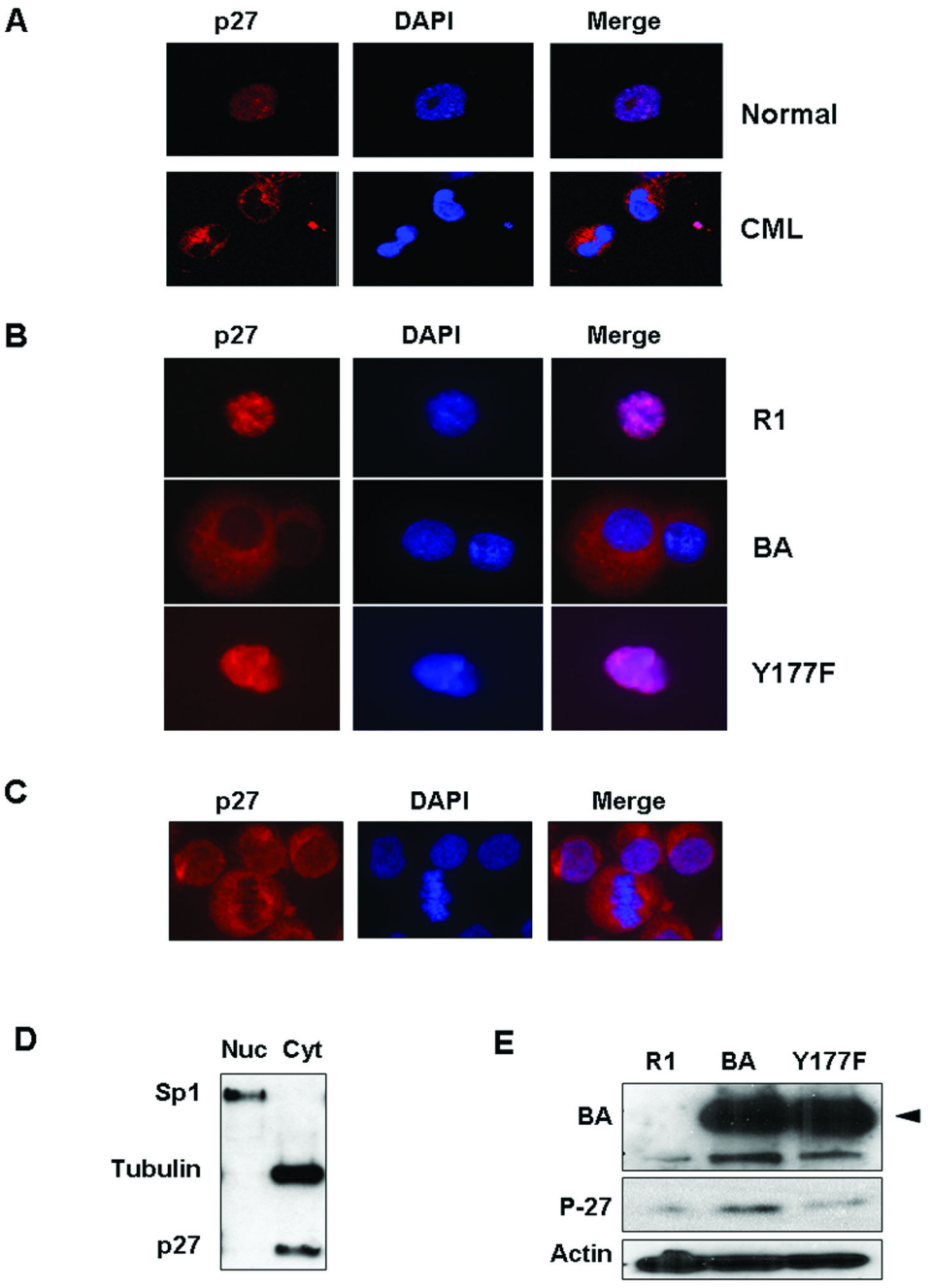


**Figure 2. Abnormal localization of p27 in CML CD34+ cells and human CD34+ cells ectopically expressing BCR-ABL; reversal by Y177F mutation**

(A) Immunofluorescence analysis of p27 in freshly isolated CML and normal CD34+ cells (n=3) labeled with anti-p27 antibody and Texas red-conjugated secondary antibody (left). Nuclei were labeled with DAPI (middle). Overlap of Texas red and DAPI staining is shown on the right. Results are representative of 3 experiments. (B) Immunofluorescence analysis of p27 in freshly sorted CD34+GFP+ cells expressing GFP alone (R1), BCR-ABL and GFP (BA) and BCR-ABL-Y177F (Y177F) labeled as described for (A) above. Results are representative of 4 experiments. (C) Immunofluorescence analysis of p27 in K562 cells

labeled as described for (A) above. (D) Nuclear and cytoplasmic fractions of K562 cells were analyzed for p27 by Western blotting. The nuclear protein Sp1 and the cytoplasmic protein tubulin were also labeled as controls for fractionation (E) Western blot analysis for BCR-ABL, p27 and Actin in GFP+CD34+ cells.

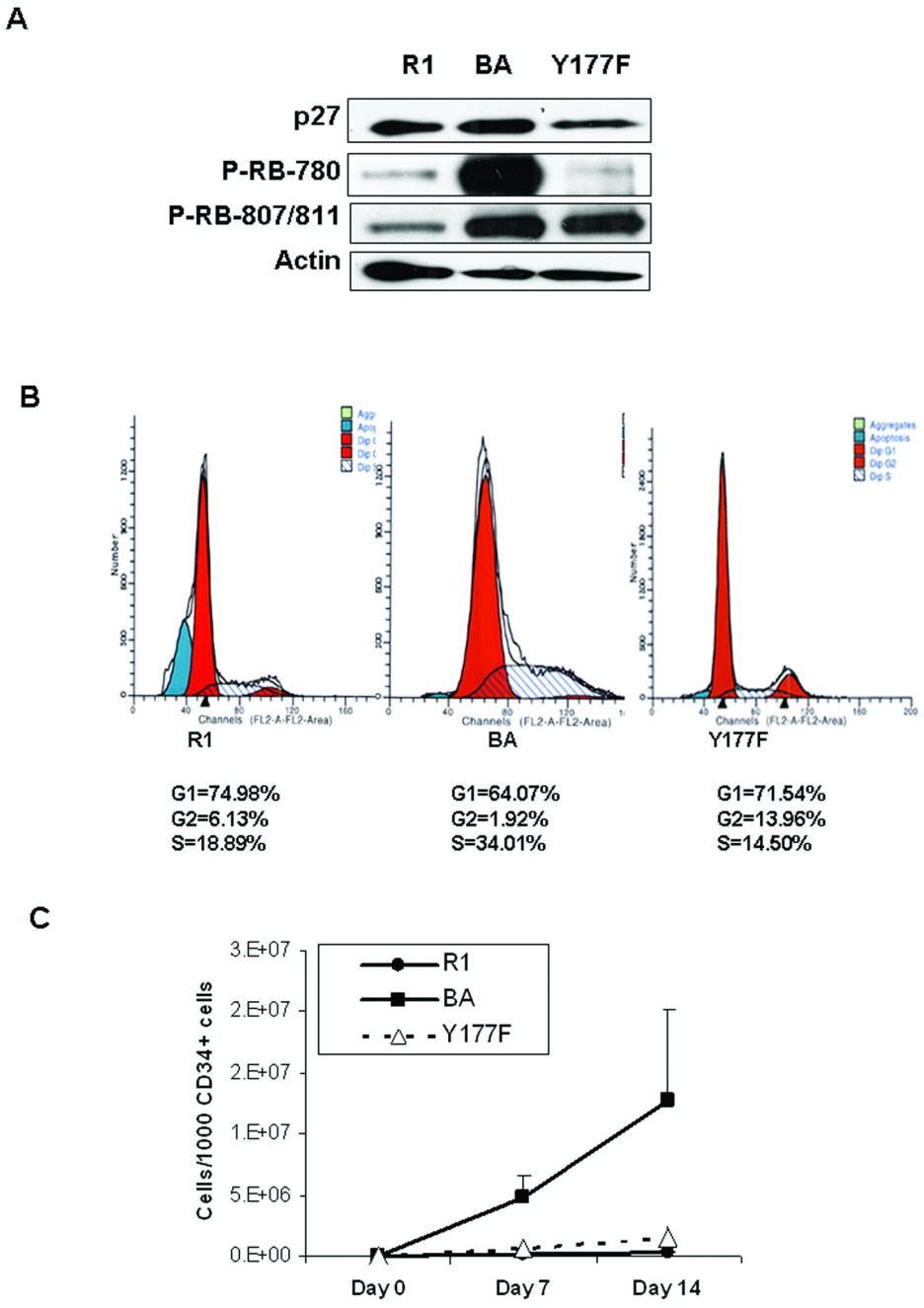


**Figure 3. Altered cell cycle regulation in human CD34+ cells ectopically expressing BCR-ABL; reversal by Y177F mutation**

(A) Western blot analysis for p27, phospho-pRB S807/S811, phospho-pRB S780 and Actin expression in CD34+ cells transduced with GFP alone (R1), BCR-ABL (BA) and BCR-ABL-Y177F (Y177F). (B) Cell cycle analysis by flow cytometry was performed on retrovirally transduced human CD34+ cells expressing GFP alone (R1, left), BCR-ABL and GFP (BA, center), and BCR-ABL Y177F and GFP (Y177F, right). (C) The same populations were cultured in GF containing media for 14 days. Cell expansion was

measured by counting the numbers at the indicated times. Results represent mean ± SEM of 7 experiments.

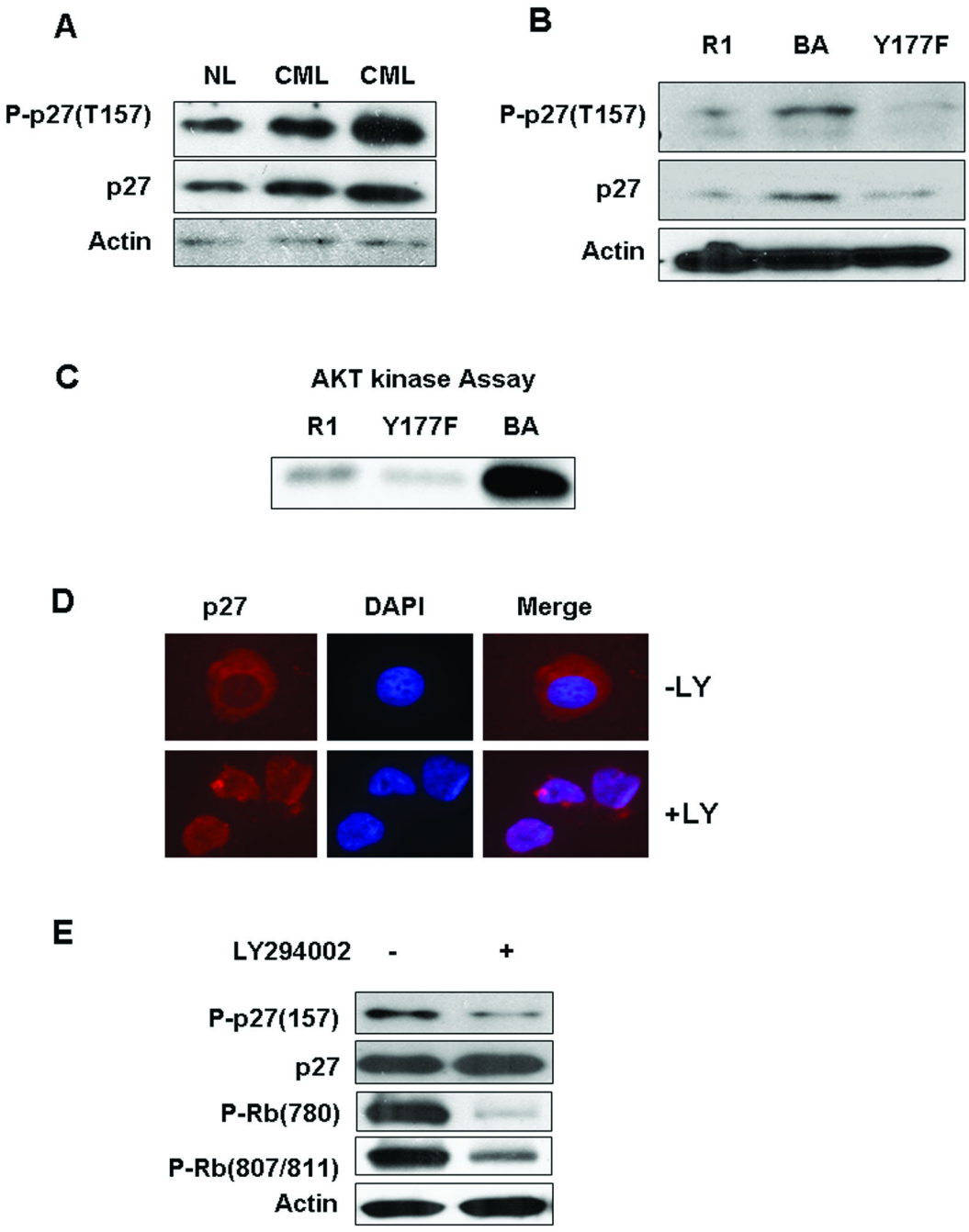


**Figure 4. Role of PI-3K signaling in p27 phosphorylation and mislocalization in CML CD34+ cells and human CD34+ cells ectopically expressing BCR-ABL**

(A) Normal (NL) or CML CD34+ cells were analyzed for p27, T157-phosphorylated p27 and Actin by Western blotting. (B) CD34+ cells expressing GFP alone (R1), BCR-ABL (BA) and BCR-ABL-Y177F (Y177F) were analyzed for p27, T157-phosphorylated p27 and Actin by Western blotting. (C) CD34+GFP+ cells were analyzed for AKT kinase activity. (D) CD34+GFP+ cells expressing BCR-ABL were cultured for 24 hours with or without LY294002 (LY) and labeled with anti-p27 antibodies and Texas red-conjugated secondary antibodies (left). Nuclei were labeled with DAPI (middle). Overlap of Texas red and DAPI

staining is shown on the right. (E) BCR-ABL expressing CD34+GFP+ cells were cultured for 24 hours with or without LY294002 and analyzed for expression of T157-phosphorylated p27, total p27, phospho-Rb and Actin by Western blotting.

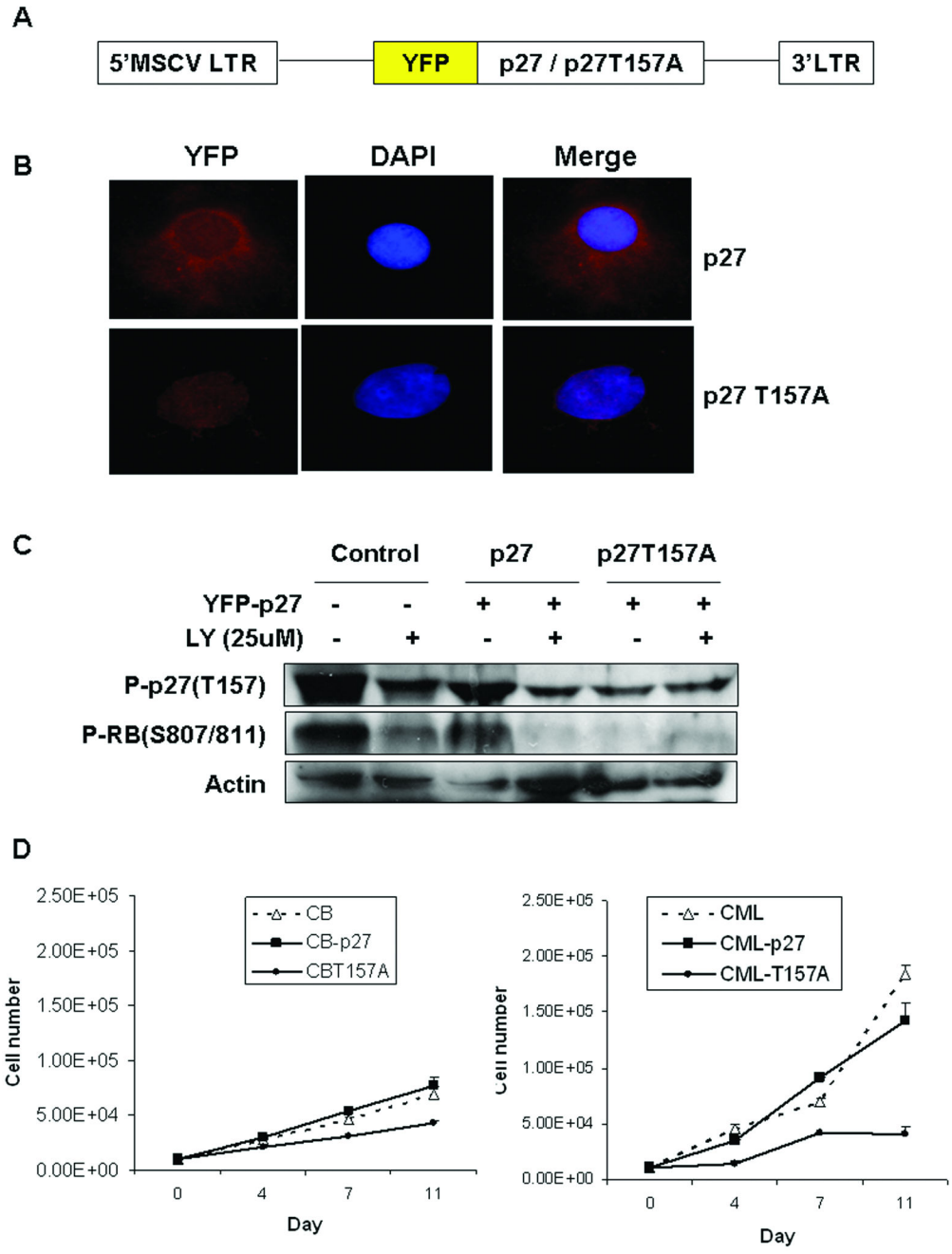


**Figure 5. Role of p27 T157 phosphorylation in altered p27 localization and function in BCR-ABL expressing human CD34+ cells**

(A) Schematic representation of MSCV vectors expressing p27 and p27T157A constructs (B) p27-YFP and p27T157A-YFP proteins were expressed in CD34+ cells from CML patients using retrovirus transduction. Cells were labeled with anti-YFP antibodies and Texas red-conjugated secondary antibodies (left). Nuclei were labeled with DAPI (middle). Overlap of Texas red and DAPI staining is shown on the right. (C) CML CD34+ cells expressing wtp27 and p27 T157A, with or without LY294002 (LY) treatment were analyzed for phosphorylated p27 T157, phospho-Rb S807/S811 and Actin by Western blotting (D)

Cord blood CD34+ cells or CML CD34+ cells transduced with p27 or p27T157A expressing vectors and non transduced cells (n=2) were cultured in GF containing media for 11 days. Cell expansion was measured by counting cell numbers at indicated times.