

# The targetable role of herpes virus-associated ubiquitin-specific protease (HAUSP) in p190 BCR-ABL leukemia

GIOVANNA CARRÀ<sup>1</sup>, CRISTINA PANUZZO<sup>1</sup>, SABRINA CRIVELLARO<sup>1</sup>, DEBORAH MORENA<sup>2</sup>,  
RICCARDO TAULLI<sup>2</sup>, ANGELO GUERRASIO<sup>1</sup>, GIUSEPPE SAGLIO<sup>1</sup> and ALESSANDRO MOROTTI<sup>1</sup>

Departments of <sup>1</sup>Clinical and Biological Sciences and <sup>2</sup>Oncology, 'San Luigi Gonzaga'  
University Hospital, School of Medicine, University of Turin, 10043 Turin, Italy

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**Abstract.** Philadelphia chromosome-positive (Ph<sup>+</sup>) acute lymphoblastic leukemia (ALL) is driven by the p190 breakpoint cluster region (BCR)-ABL isoform. Although effectively targeted by BCR-ABL tyrosine kinase inhibitors (TKIs), ALL is associated with a less effective response to TKIs compared with chronic myeloid leukemia. Therefore, the identification of additional genes required for ALL maintenance may provide possible therapeutic targets to aid the eradication of this cancer. The present study demonstrated that p190 BCR-ABL is able to interact with the deubiquitinase herpesvirus-associated ubiquitin-specific protease (HAUSP), which in turn affects p53 protein stability. Notably, the inhibition of HAUSP with small molecule inhibitors promoted the upregulation of p53 protein levels. These results suggest that HAUSP inhibitors may harbor clinically relevant implications in the treatment of Ph<sup>+</sup> ALL.

## Introduction

Identification of genes required for tumor maintenance often aids therapeutic targeting in the treatment of cancer. Philadelphia-positive (Ph<sup>+</sup>) leukemia is an example of an oncogene-addicted disease due to the essential role of breakpoint cluster region (BCR)-ABL in the development and maintenance of either chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) (1-4). However, inhibition of BCR-ABL via selective TKIs does not result in disease eradication (5). Furthermore, the response rates of patients with Ph<sup>+</sup> ALL to TKIs are much poorer than those of

patients with CML, with only 50% of patients cured by TKI plus chemotherapy and allogeneic stem cell transplantation programs (6). Therefore, it is mandatory to identify targetable pathways that synergize with BCR-ABL in the maintenance of Ph<sup>+</sup> leukemias and, in particular, ALL. The sustained inactivation of tumor suppressors is also involved in tumor maintenance. In particular, it was previously demonstrated that restoration of p53 in murine models of different forms of cancer promotes the induction of cancer-selective apoptosis without affecting normal tissues (7). This supports the concept that strategies to promote pharmacological reactivation of p53 may be important in the eradication of cancer. Recently, it was observed that BCR-ABL activates the deubiquitinase HAUSP to promote phosphatase and tensin homolog (PTEN) delocalization in CML (8). In addition to targeting PTEN (9), HAUSP is able to regulate p53 protein stability (10,11), suggesting that the BCR-ABL/HAUSP network may regulate p53 protein stability. Due to the requirement of additional therapies, particularly against Ph<sup>+</sup> ALL, the present study investigated the p190 BCR-ABL/HAUSP network and demonstrated that it affects p53 stability; therefore, this pathway may be targeted by selective inhibitors.

## Materials and methods

*Cell culture and reagents.* HEK 293T cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (Euroclone S.p.A., Pero, Italy) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and 2 mM glutamine (Euroclone S.p.A.) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The primary antibodies used were as follows: Polyclonal rabbit anti-HAUSP (cat no. sc-30164; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), monoclonal mouse anti-Myc-Tag (cat no. 2276; Cell Signaling Technology, Inc., Danvers, MA, USA), monoclonal mouse anti-phospho-tyrosine (cat no. sc-7020; Santa Cruz Biotechnology, Inc.), polyclonal rabbit anti-BCR (cat no. sc-885; Santa Cruz Biotechnology, Inc.), monoclonal mouse anti-phospho-p53 (cat no. 9286; Cell Signaling Technology, Inc.), monoclonal mouse anti-p53 (cat no. sc-98; Santa Cruz Biotechnology, Inc.) and polyclonal rabbit anti-heat shock protein 90α/β (cat no. sc-7947; Santa Cruz Biotechnology, Inc.), which was used as a loading control. Secondary goat

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*Correspondence to:* Dr Alessandro Morotti, Department of Clinical and Biological Sciences, 'San Luigi Gonzaga' University Hospital, School of Medicine, University of Turin, Regione Gonzole 10, Orbassano, 10043 Turin, Italy  
E-mail: alessandromorotti@yahoo.it

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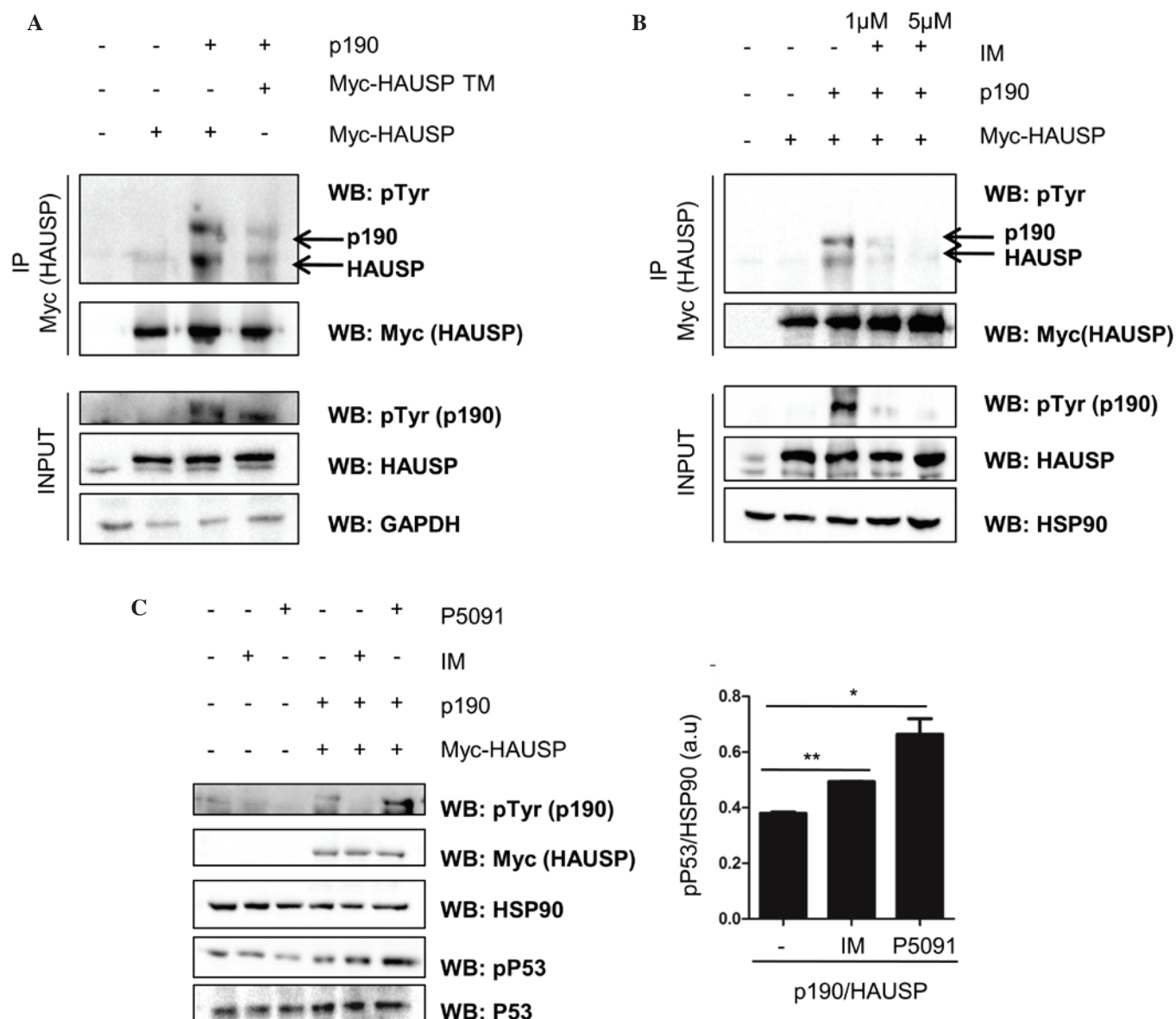


Figure 1. (A) Western immunoblotting using anti-pTyr antibody. HEK 293T cells were transfected with the HAUSP WT or HAUSP TM (Y243F/Y878F/Y947F) in the presence of p190 BCR-ABL. Immunoprecipitation was performed with anti-Myc antibodies (HAUSP). (B) Immunoprecipitation of Myc-HAUSP and anti-pTyr immunoblot following treatment with 1 or 5  $\mu$ M imatinib for 24 h; (C) Left panel: Western immunoblot of HEK 293T cells expressing p190 BCR-ABL and Myc-HAUSP. Cells were treated with P5091 and imatinib for 24 h at concentration of 4.2 and 5  $\mu$ M respectively, and were subsequently analyzed for expression of p53 and p-p53. Right panel: Quantification and statistical analysis of p-p53 protein (\*\*P=0.0018 and \*P=0.0371). P-values were calculated by a two-tailed *t*-test. p-, phosphorylated; Tyr, tyrosine; HAUSP, herpesvirus-associated ubiquitin-specific protease; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IP, immunoprecipitation; WT, wild-type; TM, triple mutant; IM, imatinib.

anti-rabbit and goat anti-mouse antibodies were from ThermoScientific (Waltham, MA, USA), cat nos. 31460 and 31430, respectively. Primary and secondary antibodies were used at 1:1000 and 1:8000 dilutions, respectively.

**Plasmid construction, transfection assay and pharmacological treatments.** Myc-Tag ubiquitin-specific-processing protease 7 (HAUSP) wild-type, Myc-Tag HAUSP triple mutant and p190 BCR/ABL plasmids were constructed as previously described (8). To perform transient transfection, the calcium phosphate transfection method was used. Briefly, for each 10-cm dish, 10  $\mu$ g DNA, 61  $\mu$ l 2 M CaCl<sub>2</sub> and enough distilled water to bring the total volume to 0.5 ml was added slowly to

0.5 ml HEPES buffered saline, which was aerated during the addition. After incubating for 20 min at room temperature, the mix was added to the plate and incubated for a further 16-24 h at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. A total of 1 or 5  $\mu$ M imatinib and 4.2  $\mu$ M P5091 was added for 24 h to inactivate p190 BCR/ABL and HAUSP, respectively. The same quantity of dimethyl sulfoxide was used for the untreated control.

**Western blot and immunoprecipitation.** Total cell extraction was performed using co-immunoprecipitation buffer [150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 50 mM HEPES (pH, 7.5), 1% Triton and 10% glycerol] supplemented with

protease inhibitor (cat no. 036K4082; Sigma-Aldrich) and a phosphatase cocktail composed of PMSF and  $\text{Na}_3\text{VO}_4$ . Following quantification by Bio-Rad Protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 30  $\mu\text{g}$  protein extract was denatured, reduced, separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. The membranes were subsequently quenched with 5% bovine serum albumin and probed overnight with primary antibodies. Protein detection was performed using peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagent (cat no. 170-5060; Bio-Rad Laboratories, Inc.), according to the manufacturer's protocol. For immunoprecipitation experiments, 500  $\mu\text{g}$  protein extracts were precleared with agarose-conjugated normal immunoglobulin G (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C and then precipitated overnight at 4°C with anti-Myc-Tag antibody along with Dynabeads® protein G. The immunoprecipitate samples were then resolved by western blot analysis. Image acquisition and analysis was performed using ImageLab software (Bio-Rad Laboratories, Inc.).

*Statistical analysis.* Two-sided Student's t-test or two way ANOVA with Bonferroni post-test were calculated using GraphPad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*Expression of HAUSP triple mutant reduces HAUSP phosphorylation.* To investigate whether p190 BCR-ABL retains the capability to interact with HAUSP and to promote HAUSP phosphorylation, p190 BCR-ABL and Myc-HAUSP-expressing vectors were transfected into HEK 293T cells. At 48 h post-transfection, the HEK 293T cells were lysed, and then immunoprecipitation with anti-Myc-Tag antibody (HAUSP) was performed. As presented in Fig. 1A, HAUSP was phosphorylated on tyrosine residues by p190 BCR-ABL. Notably, expression of the HAUSP triple mutant (Y243F/Y878F/Y947F), which bears mutants on three tyrosine residues phosphorylated by p210 BCR/ABL (8), reduced HAUSP phosphorylation levels in HEK 293T when compared to those of HAUSP WT, as observed with p210 BCR/ABL (8).

*BCR-ABL inhibition is associated with HAUSP dephosphorylation, and inhibition and deletion of HAUSP is associated with p53 destabilization.* To test the contribution of BCR-ABL tyrosine kinase in the phosphorylation of HAUSP, transfected HEK 293T cells were treated with varying concentrations of the BCR-ABL TKI, imatinib. The results demonstrated that P190 BCR-ABL inhibition, as observed with p210 BCR/ABL (8), was associated with HAUSP dephosphorylation (Fig. 1B). In addition to the ability of HAUSP to deubiquitinate PTEN and the relevance of the BCR-ABL/HAUSP/PTEN network in CML, HAUSP is also able to target p53 (10,11). In particular, HAUSP has been previously reported to interfere with the mouse double minute 2 homolog (MDM2)/p53 network causing p53 destabilization (10,11). The results of the present study were consistent with these previous findings,

demonstrating that the deletion and inhibition of HAUSP was associated with p53 upregulation (Fig. 1C).

*Imatinib and P5091 promote p53 upregulation and phosphorylation.* To test whether HAUSP inhibition is able to regulate p53 protein stability downstream of BCR-ABL, p190 BCR-ABL-transfected cells were treated with imatinib and the HAUSP inhibitor, P5091. As presented in Fig. 1C, imatinib was able to promote p53 upregulation; however, the greatest increase in p53 protein expression and phosphorylation on Ser15, a marker of functionally active p53, was observed following incubation with the HAUSP inhibitor (P5091) with subsequent apoptosis induction (data not shown).

## Discussion

Identification of signaling transduction pathways that function in the maintenance of cancer are extremely important from a therapeutic standpoint, and targeting of these pathways may aid the eradication of cancer. ALL therapy requires the detection of additional targets, not only to achieve disease abolishment, but also to achieve a more positive overall response to TKIs. The present study identified the p190-BCR-ABL/HAUSP/p53 network as a challenging, targetable pathway that promotes the regulation of p53 protein stability. The data obtained from the present study did not demonstrate whether p53 upregulation is directly regulated by HAUSP activity or is dependent on the more complex BCR/ABL/HAUSP/MDM2/p53 network, but the upregulation in p53 levels supports the assessment of HAUSP inhibitors in the clinical setting.

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