Stabilization of Notch1 by the Hsp90 Chaperone is Crucial for T-Cell Leukemogenesis

Zhaojing Wang1,2,3, Yufeng Hu3, Daibiao Xiao2, Jingchao Wang2, Chuntao Liu3, Yisheng Xu4, Xiaomeng Shi4, Peng Jiang5, Liang Huang6, Peng Li7, Hudan Liu1,2, and Guoliang Qing1,2

Abstract

Purpose: Notch1 deregulation is assuming a focal role in T-cell acute lymphoblastic leukemia (T-ALL). Despite tremendous advances in our understanding of Notch1 transcriptional programs, the mechanisms by which Notch1 stability and turnover are regulated remain obscure. The goal of the current study is to identify intracellular Notch1 (ICN1, the activated form of Notch1) binding partner(s) regulating its stability and activity.

Experimental Design: We employed immunoaffinity purification to identify ICN1-associating partner(s) and used co-immunoprecipitation to verify the endogenous protein interaction. Pharmacologic or short hairpin RNA-mediated inhibition was applied in loss-of-function assays to assess the role of tentative binding partner(s) in modulating ICN1 protein stability as well as affecting T-ALL cell expansion in vitro and in vivo. Mechanistic analysis involved protein degradation and polyubiquitination assays.

Introduction

Notch1 is a highly conserved transmembrane receptor that elicits signaling transduction required for cell proliferation, survival, and differentiation. Normally, transmembrane ligands in adjacent cells stimulate Notch1 receptor by inducing step-wise intramembrane proteolysis to produce a transcriptional effector ICN1, which specifically turns on target gene expression (1). Aberrantly activated Notch1 signaling has been implicated in a variety of tumors, including T-ALL, a malignant disorder of thymocyte progenitors (2). The central role of Notch1 in T-cell transformation was realized upon the identification of activating mutations in the Notch1 gene present in about 60% of T-ALL cases (3). Once obtaining these activating mutations, Notch1 signaling is potentiated by either eliciting ligand-independent activation or prolonging ICN1 half-life. These genetic lesions remarkably enhance ICN1 transcriptional programs and the expression of downstream genes that promote leukemogenesis such as c-Myc (4–7). Tremendous efforts have been paid in identifying downstream targets regulated by Notch1 signaling, whereas less attention is gained to understand the upstream mechanisms sustaining aberrant Notch1 activities, particularly those involved in Notch1 stabilization.

Hsp90 is an abundant, highly conserved molecular chaperone crucial for correct folding and maturation of a variety of cellular proteins that regulates cell survival, proliferation, and apoptosis. The increased expression of Hsp90, which is observed in many tumor types, reflects the efforts of malignant cells to maintain homeostasis in a hostile environment as well as tolerate alterations from numerous genetic lesions that often result in aberrant accumulations of oncoproteins. This dependence on Hsp90 appears to be a vulnerability of cancer cells and has led to the development of drugs aimed at depleting the molecular chaperone and degrading cancer proteome, leading to loss of tumor cell viability (8). Several Hsp90 inhibitors are currently being tested in both preclinical models and clinical settings (9). Many have shown promises in solid tumors (10–12) as well as

Results: We identify the Hsp90 chaperone as a direct ICN1-binding partner essential for its stabilization and transcriptional activity. T-ALL cells exhibit constitutive endogenous ICN1–Hsp90 interaction and Hsp90 depletion markedly decreases ICN1 levels. The Hsp90-associated E3 ubiquitin ligase Stub1 mediates the ensuring proteasome-dependent ICN1 degradation. Administration of 17-AAG or PU-H71, two distinct Hsp90 inhibitors, depletes ICN1, inhibits T-ALL cell proliferation, and triggers dramatic apoptotic cell death. Systemic treatment with PU-H71 reduces ICN1 expression and profoundly inhibits murine T-ALL allografts as well as human T-ALL xenografts.

Conclusions: Our findings demonstrate Hsp90 blockade leads to ICN1 destabilization, providing an alternative strategy to antagonize oncogenic Notch1 signaling with Hsp90-selective inhibitors. Clin Cancer Res; 1–13. ©2017 AACR.
hematologic malignancies (13–15). Inhibition of Hsp90 leads to degradation of its client proteins through the ubiquitin-dependent proteasome pathway (16). Downregulation of many Hsp90 substrates is in large part dependent on Stub1 activity, the U-box ubiquitin E3 ligase that interacts with the Hsp90 chaperone complex to favor substrate degradation (17).

Current treatment guidelines for patients with T-ALL include only conventional chemotherapy and overall prognosis of T-ALL remains unsatisfactory (18). Anti-Notch–targeted therapy by γ-secretase inhibitors, which inhibit Notch proteolytic processing and subsequent activation, has been launched for a long time. Unfortunately, clinical application of these inhibitors has been hampered owing to their limited efficacies and considerable side-effects (19). Thus, attempts to seek alternative approaches to Notch1 inhibition become imperative for effective T-ALL therapies.

In the current study, we identify Hsp90 as a novel ICN1-binding partner crucial for its stabilization and oncogenic function. Inhibition of Hsp90 leads to Stub1-mediated ICN1 polyubiquitination and subsequent degradation by the 26S proteasome. Two different categories of Hsp90 inhibitors, geldanamycin analogues and purine analogues, exhibit marked cytotoxicities against Notch1-dependent T-ALL cells as well as murine models. These findings suggest Hsp90 inhibition as a potential alternative approach and also a new horizon of anti-Notch1 strategy to benefit patients with T-ALL and other Notch1-addicted malignancies.

**Materials and Methods**

**Cell lines and reagents**

SIL-ALL, HPB-ALL, DND41, and Cült1 cells were kindly provided by Warren Pear (University of Pennsylvania, Philadelphia, PA; April, 2011). 293T, MOLT-4, JURKAT, and CCRF-CEM cells were purchased from ATCC (June, 2012). All cell lines were maintained as described previously (3), authenticated using the variable number of tandem repeats (VNTR) PCR assay, cultured for fewer than 6 months after resuscitation, and tested for mycoplasma contamination every 3 months using MycoAlert (Lonza).

pcDNA3-Flag-ICN1 was obtained from Warren Pear (University of Pennsylvania, Philadelphia, PA), pcDNA3-HA-Hsp90β was a gift from William Sessa (Yale University, New Haven, CT). Addgene plasmid # 22487; ref. 20, and the Myc-tagged Stub1 constructs (wild-type or mutants) were provided by Bin Li (Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China; ref. 21). For the experiments in which Hsp90β (or Stub1) was silenced in T-ALL cells, short hairpin RNA (shRNA) sequences were obtained from the collection of the RNAi Consortium (22) and cloned into a lentiviral vector PLKO.1. For ICN1 (or Stub1) overexpression in T-ALL cells, the protein coding sequence was cloned into a lentiviral vector pCDH. Primers used for shRNAs and molecular cloning are listed in Supplementary Table S1. Chemicals and antibodies used in the study are listed in Supplementary Table S2.

**Identification of ICN1-interacting partners**

293T cells stably expressing Flag-tagged ICN1 were lysed for 30 minutes and subjected to centrifugation at 12,000 × g for 15 minutes. The resulting supernatant was incubated with antibody against Flag epitope (Sigma) at 4°C for 4 hours, followed by addition of protein G-agarose (Roche; 4°C overnight). Immunoprecipitated proteasomes were washed and eluted, followed by SDS-PAGE and Coomassie blue staining (23). Gel bands were excised and digested with trypsin overnight. The resulting peptides were analyzed by the Protein Facility, Center of Biomedical Analysis at Tsinghua University (Beijing, China).

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChiP) was performed as described previously (24, 25). Briefly, SIL-ALL cells were fixed with 1% paraformaldehyde at room temperature for 10 minutes. Precleared chromatin was immunoprecipitated with antibody against cleaved Notch1 (Val1744) for 16 hours and then salmon sperm DNA–saturated protein G-agarose (Millipore) for 1 hour at 4°C. Eluted DNA was quantified by CFX Connect Real-Time PCR System (Bio-Rad) using specific primers (Supplementary Table S1).

**Mice**

Animal procedures were approved by the Animal Experimentations Ethics Committee of Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences (Guangzhou, China).

**Human T-ALL xenograft**

A total of 2.5 × 10⁶ Cült1 cells were tail vein injected into 6- to 8-week-old NOD-SCID IL2Rγ-deficient (NSI) mice (Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China) that had been subjected to 100 cGy whole-body irradiation (Precision X-ray). PU.1-HiT1 (75 mg/kg, thrice weekly) or vehicle (10 mmol/L PBS) was intraperitoneally injected into NSI mice two days after engraftment. T-ALL development in vivo was monitored by periodic blood drawings and flow cytometry analysis of human CD8 and CD45. After four weeks of treatment, mice were sacrificed and assessed for disease progression.

**Murine T-ALL transplant**

Murine T-ALL cells driven by KrasG12D/Notch1L1601P mutations were described by Chiang and colleagues (4). A total of 2.5 × 10⁶
murine T-ALL cells were injected into 6- to 8-week-old half-lethally irradiated (450 cGy) C57BL/6 mice (Beijing HFK Bioscience). Five days later, mice were intraperitoneally injected with PU-H71 (50 mg/kg, thrice weekly) or vehicle (10 mmol/L PBS). After three weeks of treatment, mice were sacrificed and assessed for leukemia progression by flow cytometry analysis of GFP + as well as CD4 “CD8” cells in the bone marrow and spleen.

**Statistical analysis**

Significance analysis between groups were performed using Student’s t-test, with P < 0.05 considered significant.

**Results**

**Identification of Hsp90 as a novel ICN1-binding protein**

To identify novel partners of ICN1, we generated 293T cells expressing Flag-tagged ICN1. Flag-bound immunoprecipitates from these cells were resolved by SDS-PAGE and visualized by expressing Flag-tagged ICN1. Flag-bound immunoprecipitates for leukemia progression by

After three weeks of treatment, mice were sacri
cence). Five days later, mice were intraperitoneally injected with

Pelocal immunoprecipitation and con

Hsp90 as a protein associated with ICN1 (Fig. 1B; Supplementary

Student’s

Statistical analysis

To validate the mass spectrometry analysis, we enforced the

To assess potential roles of Hsp90 in regulating Notch1, we

To determine the precise region(s) for Hsp90–ICN1 interaction, we expressed full-length HA-tagged Hsp90 in combination with respective Flag-tagged fragments of ICN1 in 293T cells (Fig. 1E). The N-terminal region of ICN1 (amino acids 1761–2155) containing the RAM and ANK domains did not interact with Hsp90, whereas the C-terminal region (amino acids 2156–2555) interacted as strongly as the full-length protein (Fig. 1E, lanes 1–4). As the C-terminal region of ICN1 contains the TAD domain, the PEST domain, and a linker peptide (amino acids 2425–2442; for simplicity, we named it H) between these two domains, we expressed different mutants of these interacting modules with

We postulate that stabilization of ICN1 might be a result of protection from degradation by physical association with the multicomponent Hsp90 chaperone complex. To test this and further delineate the kinetics of ICN1 degradation, we performed protein stability assays in two T-ALL cell lines (CUTLL1 and SIL-ALL) that express high levels of ICN1 and are extremely sensitive to γ-secretase inhibitor treatment. In the γ-secretase inhibitor DAPT-pretreated CUTLL1 cells, administration of 17-AAG markedly accelerated the turnover of ICN1 protein (Fig. 3A, left). Time-course analysis revealed that pharmacologic inhibition of Hsp90 significantly shortened the half-life of endogenous ICN1 from 13.8 hours to 7.4 hours (Fig. 3A, right). Similar results were obtained in SIL-ALL (Fig. 3B) as well as T6E, a murine T-ALL cell line (data not shown). Moreover, Hsp90 inhibition also significantly destabilized ectopically expressed ICN1 upon cycloheximide pretreatment to block protein synthesis; time-course analysis revealed that it significantly shortened the half-life of exogenous ICN1 from 16.3 hours to 8.5 hours (Fig. 3C). Addition of the 26S proteasome inhibitor (MG132) completely blocked ICN1 destabilization in response to 17-AAG in multiple T-ALL cell lines (Fig. 3D), further corroborating that Hsp90 regulates ICN1 expression predominantly by preventing proteosomal degradation. In support of this notion, we observed that 17-AAG treatment significantly enhanced ICN1 polyubiquitination in 293T cells (Fig. 3E, lanes 2 and 3). Collectively, these results suggest that acceleration of ICN1 degradation upon Hsp90 inactivation is strictly dependent on the ubiquitin–proteasome pathway.
ICN1 interacts with the Hsp90 chaperone.  

A, Total cell extracts prepared from 293T cells expressing Flag-tagged ICN1 or vector alone were subjected to immunoprecipitation using anti-Flag beads. Proteins were resolved by SDS-PAGE and visualized by silver staining.  

B, LC/MS-MS spectrometry of the purified Flag-ICN1–associated peptides corresponding to Hsp90.  

C, Flag-tagged ICN1 and/or HA-tagged Hsp90 were overexpressed in 293T cells and subjected to reciprocal coimmunoprecipitation (co-IP) to detect protein interaction.  

D, Coimmunoprecipitation with anti-ICN1 antibody to detect interaction between endogenous Hsp90 and ICN1.  

E, Schematic presentation of various ICN1 truncations and potential Hsp90-binding region (termed as Peptide H) used in Hsp90-binding assays. 293T cells expressing HA-tagged Hsp90 and/or various Flag-tagged ICN1 truncations were subjected to coimmunoprecipitation using anti-Flag beads (lanes 1–8). A Flag epitope followed by a tentative Hsp90-binding peptide (Peptide H) or a control peptide (Peptide Ctrl) was synthesized (ABclonal) and subjected to coimmunoprecipitation using anti-Flag beads (lanes 9 and 10).
Figure 2.
Hsp90 inactivation downregulates ICN1 expression and its transcriptional activity. A–C, ICN1 proteins were analyzed by immunoblot upon Hsp90 inhibition. ICN1 band intensities were measured by ImageJ and relative intensities are shown. A, Multiple T-ALL cells were treated with increasing concentrations of 17-AAG for 24 hours. B, T-ALL cells were treated with PU-H71 (1 µmol/L) for the indicated time points. C, Hsp90 was depleted using lentiviruses expressing shRNAs targeting Hsp90 or GFP (Ctrl). Transduced cells were purified upon puromycin (2 µg/mL) treatment for 48 hours. Before selection, initial transduction efficiencies of shGFP and shHsp90 were about 60% and 50%, respectively. D, CUTLL1 cells were treated with PU-H71 (1 µmol/L) or DMSO for 16 hours. Gene expression was quantified by qPCR. Data shown represent the means (±SD) of triplicate (**, P < 0.01). E, ChIP was performed in SIL-ALL cells treated with DMSO or 1 µmol/L PU-H71 for 16 hours. Eluted DNAs were analyzed by qPCR with primers flanking the Hes1 CSL-binding site. Data shown represent the means (±SD) of triplicates (**, P < 0.01; n.s., not significant). All the experiments were repeated at least three times.
A) DMSO and 17-AAG treatment on ICN1 and β-Actin levels with DAPT pretreatment.

B) DMSO and 17-AAG treatment on ICN1 and β-Actin levels with DAPT pretreatment.

C) DMSO and 17-AAG treatment on Flag-ICN1 and β-Actin levels with CHX pretreatment.

D) Western blots showing ICN1 and β-Actin levels with 17-AAG and MG132 treatments.

E) IP-Flag showing ICN1 and Ubi levels.
The ubiquitin E3 ligase Stub1 negatively regulates ICN1

The observation that enhanced ICN1 polyubiquitinylation upon 17-AAG treatment prompted us to identify the E3 ligase that mediates ICN1 degradation. In this regard, the Hsp90-associated ubiquitin E3 ligase Stub1 (Sip1 homology and U-box containing protein 1, also called CHIP) is recruited to induce proteasomal degradation of misfolded or aggregated molecules (17). We depleted Stub1 by a specific shRNA and then subjected these cells to 17-AAG treatment. In comparison with the mock treatment, Stub1 depletion largely prevented 17-AAG–induced ICN1 degradation in HPB-ALL and MOLT-4 cells (Fig. 4A). Consistent with prior findings that Stub1 reduces chaperone efficiency and induces substrate degradation (17, 28, 29), ectopic expression of Stub1 markedly diminished endogenous ICN1 expression in 293T and multiple T-ALL cells even when Hsp90 inhibitors were absent (Fig. 4B). Exogenous Stub1 expression markedly accelerated Flag-tagged ICN1 degredation upon 17-AAG treatment of 293T cells (Fig. 4C); time-course analysis revealed that it significantly shortened the half-life of endogenous ICN1 from 9.0 hours to 4.2 hours (Fig. 4D). Similar to Fbw7, a well-characterized ICN1 E3 ligase (30, 31), wild-type Stub1 significantly inhibited ICN1-induced luciferase activity. In contrast, Stub1 E3 ligase-inactive H260Q mutant or substrate-binding–deficient K30A mutant failed to induce any noticeable effects (Fig. 4E). Coimmunoprecipitation demonstrated a robust physical interaction of ICN1 and Stub1 when coexpressed in 293T cells (Fig. 4F). As such, only wild-type Stub1, but not its inactivating H260Q or K30A mutant, resulted in robust ICN1 polyubiquitination, and Hsp90 inhibition by PU-H71 further enhanced polyubiquitination densities (Fig. 4G). In sum, these results identify Stub1 as the E3 ligase that is largely responsible for ICN1 degradation via the ubiquitin–proteasome pathway upon Hsp90 inhibition in T-ALL cells.

Hsp90 inhibition induces T-ALL cell apoptosis in vitro and impedes xenograft growth in vivo

To determine the biological outcomes of Hsp90 inhibition in T-ALL, we first assessed the effect of 17-AAG or PU-H71 on survival of multiple T-ALL cell lines that harbor Notch1 gain-of-function mutations and rely on Notch1 activity for efficient expansion. In T-ALL cell lines, we tested 17-AAG- or PU-H71–induced robust apoptosis (Supplementary Fig. S1A) and effectively decreased cell viability in a dose-dependent manner (Fig. 5A). Notch1 activation was shown to sustain aerobic glycolysis (the Warburg effect) in T-ALL cells (32, 33). Consistently, we found that Hsp90 pharmacologic inhibitors, which caused ICN1 depletion, markedly inhibited glucose uptake and subsequent lactate secretion in HPB-ALL and CUTLL1 cells (Supplementary Fig. S1B). To test whether ICN1 degradation contributes to the antitumor effects of Hsp90 inhibition in T-ALL cells, we overexpressed ICN1 in HPB-ALL cells, and found that ectopic ICN1 expression partially, but significantly, rescued 17-AAG–induced growth inhibition (Fig. 5B), arguing that ICN1 degradation is an important route that mediates the cytotoxic effects of Hsp90 inhibition.

To translate the effects of Hsp90 deficiency in vitro into an in vivo setting, we assessed leukemia burden in T-ALL xenograft–bearing mice with or without PU-H71, which had shown great efficacies in various preclinical tumor models (15, 34–36), and is currently under clinical examination (27). For this, CUTLL1 cells were intravenously injected into immunodeficient NSG mice. Engrafted mice were randomized and respectively treated with 75 mg/kg PU-H71 or vehicle. This dose is well tolerated with minimal body weight loss (Supplementary Fig. S2A), consistent with previous reports that chronic PU-H71 (75 mg/kg) therapy is not associated with significant toxicities (27, 34). The percentage of human CD45+CD8+ leukemic cells was significantly reduced in bone marrow and spleen of each PU-H71–treated mouse (Fig. 5C; Supplementary Fig. S2B), resulting in bones with more reddish color and spleens with much smaller sizes (Fig. 5C; Supplementary Fig. S2C). Hematoxylin and eosin (H&E) staining showed that, in comparison with vehicle treatment, PU-H71 significantly reduced lymphoblastic leukemia burdens in bone marrow and subsequent leukemia cell infiltration into spleens and livers (Supplementary Fig. S3). Consistent with the in vitro results shown in Figs. 2B and 5A, splenocytes from PU-H71–treated mice exhibited reduced ICN1 and PCNA (a marker indicating cell proliferation) IHC staining (Fig. 5D). As such, Hsp90 inactivation significantly decreased Notch1 target gene expression in sorted human CD45+ splenocytes (Supplementary Fig. S2D), arguing that PU-H71 functions, in part, through Notch1 downregulation. Administration of PU-H71 did not substantially improve overall survival (Supplementary Fig. S2E), suggesting that PU-H71, as a single agent, is insufficient for a prolonged survival in our disease model. Most likely, a drug combination with a standard chemotherapy would achieve a more desirable outcome.

PU-H71 impedes T-ALL leukemia progression in a murine allograft model

Notch1 gain-of-function mutations at the heterodimerization domain are weak alleles to elicit leukemogenesis but capable of accelerating T-ALL development in K-rasG12D transgenic background (4). We obtained these primary T-ALL cells driven by K-rasG12D and Notch1L1601P mutants (Notch1L1601P is expressed in MigR1 virus with GFP as a surrogate marker) and also found that Hsp90 antagonism decreased ICN1 levels (Fig. 6A) and its transcriptional activity (Supplementary Fig. S4A). These murine
T-ALL cells were then intravenously injected into half-lethally irradiated C57Bl/6 mice for a secondary transplant. After three weeks of either vehicle or 50 mg/kg PU-H71 treatment, mice were sacrificed and assessed for in vivo leukemia cell expansion (Fig. 6B). PU-H71 treatment significantly reduced the GFP− leukemic cell percentages in the bone marrows and spleens (Fig. 6C and D). Murine T-ALL cell population characterized by CD4+ CD8− staining were markedly reduced in PU-H71−treated mice compared with the vehicle-treated group (Fig. 6E and F). Moreover, PU-H71 treatment ameliorated splenomegaly (Fig. 6G) and leukemia cell infiltration into spleens (Fig. 6H). Again, the Notch1 targets were significantly downregulated in GFP− splenocytes from PU-H71−injected mice (Supplementary Fig. S4B). Altogether, these results show that pharmacologic inactivation of Hsp90 reduces leukemia burden in vivo and provide proof-of-concept in administration of Hsp90 inhibitors as a potential therapeutic regimen for Notch1-addicted T-ALLs.

**Discussion**

Much progress has been made in identifying transcriptional oncogenic programs activated by Notch1 during T-ALL pathogenesis. Yet not much is known about the molecular mechanisms sustaining the aberrant Notch1 activities, especially those critical for Notch1 stabilization. In the current study, we show that Hsp90 is responsible for aberrant ICN1 accumulation in T-ALL cells. Upon Hsp90 inhibition, the ubiquitin E3 ligase Stub1 mediates ICN1 polyubiquitination and subsequent proteasome-dependent degradation. These data describe a previously unsuspected pathway, amenable to pharmacologic manipulation, which mediates ICN1 stability.

Conventional chemotherapy is still the mainstay treatment guideline for patients with T-ALL and overall prognosis of T-ALL remains unsatisfied (2, 37, 38). Effective targeted therapies are currently lacking. Inhibition of Notch by γ-secretase inhibitors have not achieved impressive efficacy and yielded considerable side-effects (19). In this report, we show that Hsp90 inhibition depletes ICN1 expression and two distinct Hsp90 inhibitors (17-AAG and PU-H71) demonstrate efficacy in Notch1-dependent T-ALL cells and murine models. These effects were associated with dose-dependent, potent in vitro and in vivo inhibition of Notch1 activation (via ICN1 degradation) and downstream target expression. In addition to T-ALL, aberrant Notch activation has been identified in ovarian cancer, breast cancer, lung carcinoma, and cancers of the pancreas and prostate (39). Although not yet FDA approved, the clinical development of Hsp90 inhibitors is making steady progress. There are currently more than twenty active clinical trials involving Hsp90 inhibitors. Conceivably, these Hsp90 inhibitors would act as potential, alternative therapeutic regimens to benefit patients with Notch-dependent malignancies. More interestingly, we have identified the particular ICN1 sequence (peptide H), which mediates the interaction between ICN1 and Hsp90. In principle, cellular delivery of this peptide would block ICN1−Hsp90 interaction and lead to selective ICN1 degradation, which holds great promise for therapeutic purposes in Notch-addicted tumors.

Stub1 is a cochaperone that interacts with Hsp70/90 and substrates. Stub1 remodels Hsp90 machinery and induces proteasome-mediated substrate degradation when Hsp90 is inactivated (17). Consistently, we demonstrate that 17-AAG−induced ICN1 turnover is largely blocked when Stub1 is silenced. It is well supported that Stub1 modulates protein triage decisions that regulate the balance between protein folding and degradation for chaperone substrates. When overexpressed, Stub1 negatively regulates Hsp90 chaperone function evidenced by inhibiting the glucocorticoid receptor, a well-characterized Hsp90 client (29). Similarly, here we show enhanced expression of Stub1 promotes ICN1 polyubiquitination and subsequent protein degradation, suggesting a critical role of Stub1 in the triage decision of ICN1 protein folding or degradation. This observation raises a possibility that manipulation of Stub1 levels may affect T-ALL cell growth. Indeed, depletion of Stub1 significantly enhanced, whereas its overexpression inhibited T-ALL cell growth in vitro (Supplementary Fig. S5). Considering the negative roles of Stub1 in regulation of oncogenic substrates, it is unsurprising that Stub1 could play a tumor-suppressive role (40−42). Whether manipulation of Stub1 offers a therapeutic opportunity in cancer treatment awaits more vigorous in vivo investigation using complementary disease models.

As inhibition of Hsp90 simultaneously downregulates multiple oncogenic client proteins crucial for cell viability and tumor development, it is likely that the effects of 17-AAG and PU-H71 result from inhibition of multiple target proteins in addition to ICN1. Several oncogenic Hsp90 substrates, including AKT (43), JAK (36), and Tyk2 (44, 45), also play important roles in sustaining the aberrant Notch1 activities, especially those critical in Notch-addicted tumors.

**Figure 4.** Stub1 promotes ICN1 ubiquitination and degradation. A, HPB-ALL and MOLT-4 cells were transduced with lentivirus carrying shRNAs against the control GFP or Stub1. Transduced cells were purified upon puromycin (2 μg/mL) treatment for 48 hours. Before selection, initial transduction efficiency of shGFP or shStub1 was about 50%. Cells were subsequently treated with 17-AAG (1 μmol/L) for 24 hours. ICN1 and Stub1 protein levels were assessed by immuno blotting with β-actin as a loading control. B, 293T, HPB-ALL and CUTLL1 cells were infected with lentiviruses expressing pCDH-Stub1 or empty vector for 48 hours. Initial transduction efficiencies of pCDH and pCDH-Stub1 were about 40% and 30%, respectively. After 2 μg/mL puromycin selection for 48 hours, ICN1 and Stub1 protein levels were assessed by immunoblot with β-actin as a loading control. C, 293T cells were transfected with Myc-tagged Stub1 and/or Flag-tagged ICN1 for 48 hours. Cells were pretreated with cycloheximide (100 μg/mL) for 2 hours, followed by 17-AAG treatment (1 μmol/L) for indicated time points. ICN1 and Stub1 protein levels were assessed by immunoblotting with β-actin as a loading control. D, ICN1 band intensities were quantified, normalized to β-actin, and then normalized to t = 0 controls. Data shown are averages of three independent experiments. E, Hes1 promoter luciferase activity was determined in 293T cells expressing Flag-ICN1, Flag-ICN1/Myc-Stub1 (WT or H260Q, K30A mutant) or Flag-ICN1/Myc-Fbw7. Data shown represent the means ± SD of triplicates (*, P < 0.05; ***, P < 0.001). n.s., means not significant). These experiments were repeated at least three times. F, ICN1 and Stub1 protein interaction. Lysates from 293T cells overexpressing Flag-tagged ICN1 and Myc-tagged Stub1 were subjected to immunoprecipitation using anti-Flag antibody, and the coprecipitated Stub1 was detected by anti-Myc antibody. G, in vivo ICN1 polyubiquitination assay. 293T cells were transfected with plasmids expressing HA-tagged ubiquitin, Flag-ICN1, and Myc-tagged Stub1 (wild-type or mutants) for 12 hours, followed by PU-H71 (1 μmol/L) or DMSO treatment for 16 hours. MG132 (10 μmol/L) was included 5 hours prior to harvest; cells were subsequently lysed in RIPA buffer (24). Ubiquitin-conjugated ICN1 proteins were immunoprecipitated with Flag-tag antibody and subjected to immunoblot with ubiquitin antibody.

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Hsp90 inhibition exhibits antileukemia effects in T-ALL cells and human T-ALL xenografts. A, Assessment of T-ALL cell viability. Notch1-dependent T-ALL cell lines were treated with 17-AAG or PU-H71 at the various concentrations for 24 hours. Cell viability was determined using CCK8 assay kit. Data shown represent the means (±SEM) of triplicates (***, P < 0.01). These experiments were repeated at least three times.

B, HPB-ALL cells transduced with pCDH or pCDH-ICN1 were subjected to DMSO or 17-AAG (1 μmol/L) treatment as indicated. Initial transduction efficiencies of pCDH and pCDH-ICN1 were about 40% and 25%, respectively. After puromycin (2 μg/mL, 48 hours) selection, viable cells were counted and plotted as shown. Data shown represent the means (±SEM) of triplicates (***, P < 0.01). ICN1 overexpression was confirmed by immunoblotting.

C, In vivo CUTLL1 cell expansions in vehicle or PU-H71–treated mice were presented as human CD45+CD8+ percentages in the bone marrow (BM) or spleen. Representative bone and spleen images from treated or untreated cohorts are shown at the bottom. D, IHC staining of ICN1 and PCNA in the spleens from vehicle or PU-H71–treated mice. Representative images are shown (top, 200×; bottom, 1,000×). Relative ICN1 or PCNA staining was quantified and presented as mean ± SD, 5 mice per group (***, P < 0.01).
Figure 6. PU-H71 reduces tumor burden in a murine T-ALL allograft. **A**, Murine T-ALL cells derived from T-ALL mice driven by K-rasG12D and Notch1L1601P mutants were treated with PU-H71 (1 μmol/L) for the indicated time points. ICN1 levels were analyzed by immunoblotting and relative band intensities are shown. **B**, Schematic presentation of secondary transplant of T-ALL cells derived from T-ALL mouse model driven by K-rasG12D and Notch1L1601P mutants. Five days post-transplantation, PBS (n = 5) or PU-H71 (n = 5) was administered thrice weekly. After three weeks of treatment, mice were sacrificed for analysis. **C and D**, Leukemia penetrations in the spleen and bone marrow were assessed by flow cytometry analysis of GFP fluorescence. Representative plots (C) and GFP⁺ percentages from all mice (D) are shown. **E and F**, Leukemia penetrations in the spleen and bone marrow were analyzed by flow cytometry analysis of CD4⁺CD8⁺ populations. Representative plots (E) and CD4⁺CD8⁺ percentages from all mice (F) are shown. **G**, Spleen weights (top) and representative spleen images (bottom) are presented. **H**, Representative spleen H&E stains are shown (top, 200×; bottom, 1,000×). *, P < 0.05; **, P < 0.01.
roles in T-ALL pathogenesis. By adding an important new member in our data, we provide a rationale for immediate clinical development of Hsp90 inhibitors in treating T-ALL and other Notch1-addicted malignancies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: G. Qing
Development of methodology: Z. Wang, Y. Hu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Wang, Y. Hu, D. Xiao, P. Li
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Wang, Y. Hu, D. Xiao, H. Liu, G. Qing
Writing, review, and/or revision of the manuscript: Z. Wang, Y. Hu, H. Liu, G. Qing

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Zhaojing Wang, Yufeng Hu, Daibiao Xiao, et al.

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