

## Phosphatase PRL2 promotes oncogenic NOTCH1-induced T cell leukemia

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Acute lymphoblastic leukemia (ALL) is a heterogeneous group of hematologic malignancies that arise from clonal proliferation of immature lymphoid cells in the bone marrow, peripheral blood, and other organs.<sup>1</sup> T-cell acute lymphoblastic leukemia (T-ALL) is characterized by outgrowth of immature T-cells.<sup>1</sup> Gain-of-function mutations in NOTCH1 are common in T-ALL,<sup>2</sup> making this receptor a promising target for drugs such as  $\gamma$ -secretase inhibitors (GSIs), which can block a proteolytic cleavage required for NOTCH1 activation.<sup>3</sup> However, the enthusiasm for these therapies has been tempered by tumor resistance and paucity of information on the oncogenic programs regulated by oncogenic NOTCH1.<sup>3,4</sup> Therefore, the purpose of this study was to identify oncogenic NOTCH1-mediated molecular and genetic changes that contribute to T-ALL for future targeted therapies in order to improve leukemia treatment.

The phosphatase of regenerating liver (PRL) family of phosphatases, consisting of PRL1, PRL2, and PRL3, represents an intriguing group of proteins that are currently being validated as potential biomarkers and therapeutic targets in human cancer, including breast cancer, ovarian cancer, and lung cancer.<sup>5</sup> Notably, recent findings indicate that PRLs play important roles in the pathogenesis of hematological malignancies.<sup>6</sup> For instance, PRL3 is highly expressed in BCR-ABL positive ALL, which may be responsible for the aggressive phenotype of the disease.<sup>7</sup> *PRL2* is located on human chromosome 1p35, a region often rearranged or amplified in malignant lymphoma and B-cell chronic lymphocytic leukemia (B-CLL).<sup>5</sup> *PRL2 mRNA* is also highly expressed in primary human acute myeloid leukemia (AML) and T-ALL cells, suggesting that PRL2 may play an important role in the pathogenesis of T-ALL.<sup>8,9</sup> We recently generated *Prl2* deficient mice and found that PRL2 is required for extra-embryonic development by negatively regulating PTEN, thereby activating the PI3K-Akt pathway.<sup>10</sup> In a previous study, we observed that PRL2 enhances hematopoietic stem and progenitor cell (HSPC) proliferation through mediating SCF/KIT signaling.<sup>11</sup> These findings suggest that PRLs may represent novel therapeutic targets for the treatment of hematological malignancies.<sup>6-11</sup>

To determine the functional requirement for PRL2 in the pathogenesis of T-ALL, we analyzed PRL2 expression in a gene expression profiling data set reported by Haferlach and colleagues.<sup>9</sup> We found that PRL2 is highly expressed in both human B-ALL and T-ALL patient cells compared to normal control cells (Figure 1a). Given that PRL2 is important for the proliferation and survival of hematopoietic stem and progenitor cells,<sup>11</sup> we determined the effect of genetic inhibition of PRL2 on human T-ALL cell proliferation by introducing shRNA targeting PRL2 into human T-ALL cells using lentiviruses. We found that knockdown of PRL2 decreased the proliferation and survival of human T-ALL cells (Figures 1b and 1c; Supplementary Figures S1a and S1b). We also found that ectopic expression of a dominant-negative PRL2 mutant (PRL2-CSDA) decreased the proliferation of Molt4 cells compared to mock transduced cells (Supplementary Figure S1c).<sup>12</sup>

In a previous study, we conducted an unbiased high-throughput small-molecule library screen and identified several compounds capable of inhibiting PRL activity.<sup>13</sup> The lead compound (Cmpd-43) displayed a respectable pharmacokinetic profile in mouse with a plasma compound exposure  $C_{\max}=0.3$  mM and a half-life  $t_{1/2}=15.8$  h at a single 20 mg/kg intraperitoneal dosage. Intraperitoneally injection of 15 mg/kg of PRLi daily for 3 weeks exhibited no toxicity and body weight and weights of major organs (liver, spleen, and kidney) were comparable to control mice.<sup>13</sup> We named the compound Cmpd-43 as PRL inhibitor (PRLi). To determine the effect of pharmacological inhibition of PRL2 on human T-ALL cells, we treated several human T-ALL cell lines with PRLi and measured cell viability. We found that pharmacological inhibition of PRL2 activity decreased proliferation and enhanced apoptosis in a dosage-dependent manner (Figures 1d and 1e). PRLi appears to be specific to leukemia cells and did not affect the viability of human cord blood mononuclear cells and HSPCs (Supplementary Figures S1d, S1e and S1f). Given that PRLi specifically blocks the PRL1-induced cell proliferation and migration through attenuation of both ERK1/2 and AKT activity,<sup>13</sup> we treated two human T-ALL cell lines with PRLi and then measured the level of phosphorylated ERK using western blot analysis. We found that PRLi treatment resulted in decreased ERK phosphorylation in both Loucy and CEM cells (Figure 1f),

suggesting that PRLi can inhibit ERK activation in leukemia cells. These results demonstrate that PRL2 is important for the proliferation and survival of human T-ALL cells.

More than 90% of human T-ALL show signs of constitutive NOTCH1 pathway activation and majority of human T-ALL lines are addicted to NOTCH1 function.<sup>4</sup> Indeed, we found that PRL2 is a direct target of NOTCH1 signaling in mouse T cell progenitors (M.K. and Y.L. unpublished data). In order to test whether PRL2 is important for oncogenic NOTCH1 induced T-ALL *in vivo*, we transduced lineage<sup>-</sup> bone marrow progenitors from wild-type and *Prl2* null mice with retrovirus encoding the constitutively active intracellular domain of NOTCH1 (NOTCH1-ICN1-EGFP). These cells were then transplanted into lethally irradiated recipient mice, and we monitored for the presence of CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) leukemic cells in the peripheral blood.<sup>14</sup> We found that the peripheral blood of control animals contained high number of donor-derived CD4<sup>+</sup>CD8<sup>+</sup> DP cells at 5 and 7 weeks after transplantation, while only a minute number of leukemic cells were observed in recipient of *Prl2* null cells (Figure 2a). The development of T-ALL in host that received wild-type cells transduced with NOTCH1-ICN1 retrovirus was rapid, with all control animals succumbing to the disease and died 9 weeks following transplantation (Figure 2b). In contrast, animals repopulated with *Prl2* null cells displayed significant protection from disease, with majority of animals remaining disease free 18 weeks following transplantation (Figure 2b). All control animals show enlarged spleen compared with recipient mice repopulated with PRL2 null cells (Figure 2c and Supplementary Figure S2a). *Prl2*<sup>-/-</sup> cells expressing NOTCH1-ICN1 showed long-term myeloid cell, B cell, CD4<sup>+</sup>, and CD8<sup>+</sup> T cell engraftment in the surviving recipients (Supplementary Figure S2b). These results demonstrated that PRL2 is essential for the initiation and/or maintenance of T-ALL-induced by oncogenic NOTCH1.

To determine whether PRL2 is essential for T-ALL maintenance, we introduced NOTCH1-ICN1 into *Prl2*<sup>+/+</sup> and *Prl2*<sup>-/-</sup> fetal liver cells using retroviruses and transplanted transduced cells (GFP<sup>+</sup>) into lethally irradiated recipient mice. All recipient mice repopulated with *Prl2*<sup>+/+</sup> and *Prl2*<sup>-/-</sup> cells development leukemia with similar latency and penetrance (data not shown). Recently, the leukemia

initiating cells (LICs) in a mouse model of T-ALL were characterized as c-Kit<sup>+</sup> cells.<sup>15</sup> We found that the frequency of leukemia-initiating cells (Kit<sup>+</sup>CD44<sup>+</sup>CD25<sup>+</sup>) in the spleen of leukemic mice are comparable between *Prl2*<sup>+/+</sup> and *Prl2*<sup>-/-</sup> groups (Figure 2d). We then transplanted 20,000 GFP<sup>+</sup> cells purified from the spleen of primary recipients into secondary recipients and monitor leukemia development. Recipient mice repopulated with *Prl2*<sup>-/-</sup> cells show delayed disease onset and extended survival compared to that of the *Prl2*<sup>+/+</sup> cells (Figure 2e). To determine the role of PRL2 in leukemia-initiating cell maintenance, we performed limiting dilution transplantation assays and transplanted 40 or 200 leukemia-initiating cells (Kit<sup>+</sup>CD44<sup>+</sup>CD25<sup>+</sup>) purified from the spleen of primary recipients that have developed leukemia into lethally irradiated recipient mice. While most recipient mice repopulated with *Prl2*<sup>+/+</sup> cells expressing NOTCH1-ICN1 developed leukemia and died within 40 days, none of the recipient mice repopulated with *Prl2*<sup>-/-</sup> cells developed leukemia (Figure 2f). Further, LICs were not detectable in the spleen of recipient mice repopulated with *Prl2*<sup>-/-</sup> LICs (Figure 2g). It appears that PRL2 is important for the ability of LICs, defined immunophenotypically by flow cytometry, to recapitulate leukemia in secondary hosts rather than maintaining the number of LICs. Thus, these data demonstrate that PRL2 is important for the maintenance of T-ALL mediated by oncogenic NOTCH1.

DELTA/ NOTCH1 association is one of the most important signals provided by the thymic environment to initiate T cell differentiation.<sup>16</sup> Given that NOTCH1 signaling upregulates c-Kit expression in early T cell progenitors (ETPs) (M.K. and Y.L. unpublished data), we hypothesized that PRL2 may promote the pathogenesis of T-ALL through maintaining c-Kit<sup>+</sup> LICs. To test this hypothesis, we introduced NOTCH1-ICN1 into Lin<sup>-</sup>Sca1<sup>+</sup> BM cells isolated from *Prl2*<sup>+/+</sup> and *Prl2*<sup>-/-</sup> mice and then cultured infected cells without stroma to examine cell differentiation and expansion. While *Prl2*<sup>+/+</sup> Lin<sup>-</sup>Sca1<sup>+</sup> cells expressing NOTCH1-ICN1 showed expansion, *Prl2*<sup>-/-</sup> Lin<sup>-</sup>Sca1<sup>+</sup> cells failed to proliferate under this condition (Supplementary Figure S2c). We found the level of c-Kit proteins in *Prl2*<sup>+/+</sup> cells expressing NOTCH1-ICN1 was significantly higher than that of the *Prl2*<sup>-/-</sup> cells both at day 11 and day 17 (Supplementary Figure S2d). Consistent with these observations, we observed

high level of PRL2 mRNAs and proteins in the spleen of recipient mice transplanted with Lin<sup>-</sup> cells expressing NOTCH1-ICN1 (Supplementary Figures S2e and S2f). Further, treatment of some human T-ALL cells with  $\gamma$ -secretase inhibitor (GSI) decreased the level of PRL2 proteins (Supplementary Figure S2g). Thus, our data suggest that oncogenic NOTCH1 may promote T-ALL development through sustaining PRL2 and c-Kit expression.

To date, very little information exists in the literature on the role of PRL2 in human T-ALL.<sup>5,6</sup> In this study, we have identified a critical role for PRL2 phosphatase in the proliferation and survival of human T-ALL cells. Further, we demonstrated that PRL2 is important for the leukemogenic potential of oncogenic NOTCH1 *in vivo*. Our findings suggest that pharmacological inhibition of PRL2 holds potential as a novel therapy for T cell acute lymphoblastic leukemia, and might also be applicable to the treatment of other human cancers.

## **CONFLICT OF INTEREST**

The authors declared that no conflict interest exists.

## **ACKNOWLEDGEMENTS**

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## **AUTHOR CONTRIBUTIONS**

MK, YB, ZYZ, and YL. Designed the research. MK, YB, SC, RG, CY, and WC. Performed the research; MK and YL. Analyzed the data and performed the statistical analysis. AAC and JC. Provided reagents to the study. MK and YL. Wrote the manuscript. All authors read, comment on, and approved the manuscript.

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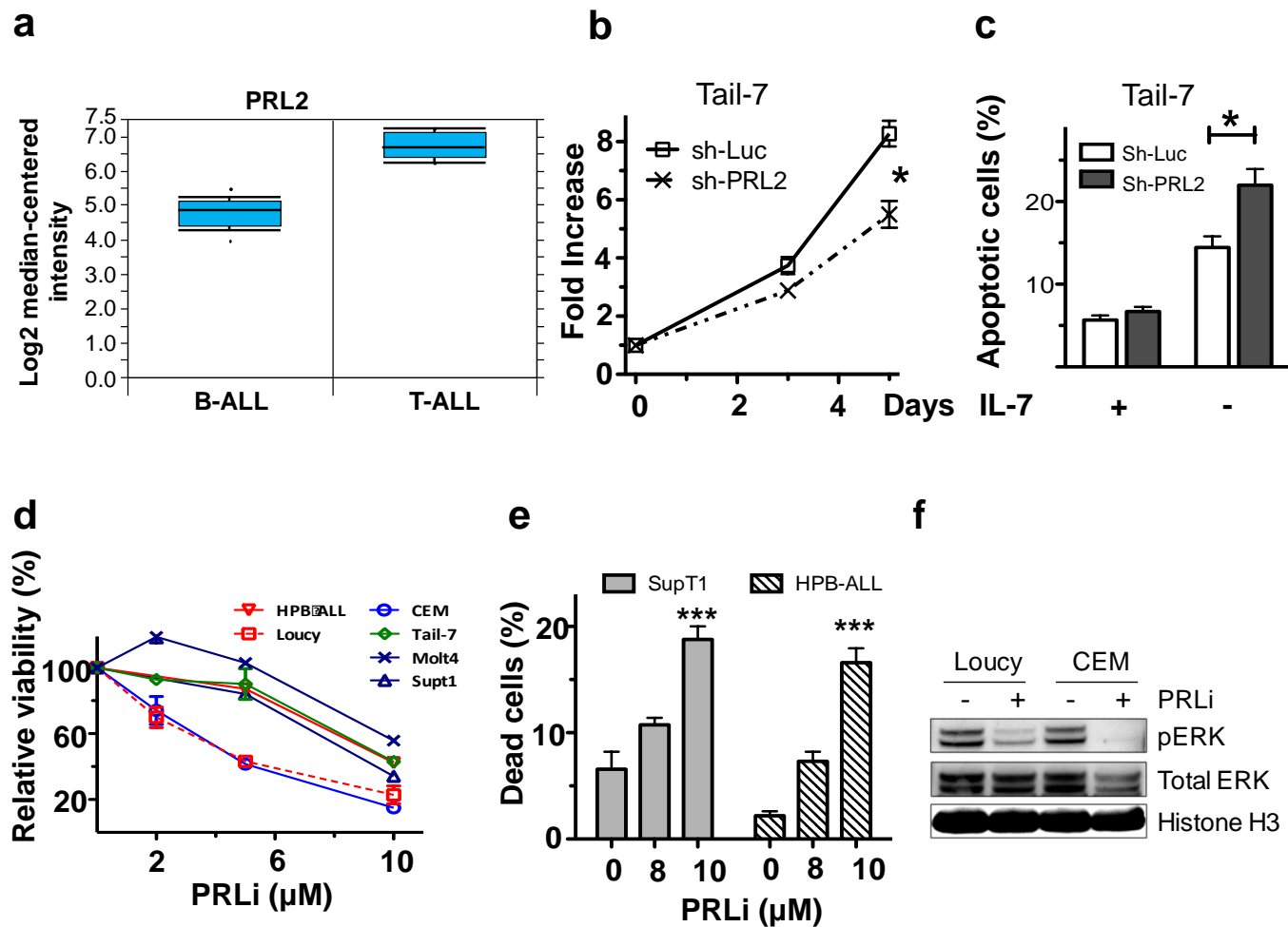
## FIGURE LEGENDS

**Figure 1.** PRL2 is important for the proliferation and survival of human T-ALL cells. **(a)** PRL2 is highly expressed in both primary human B-ALL and T-ALL cells (Normal control cells were set to 1).<sup>8</sup> **(b)** Tail-7 cells were transduced with lentiviruses expressing control or PRL2 shRNA. The proliferation of transduced cells (GFP<sup>+</sup>) was measured over time (\*p<0.05, n=3). **(c)** Tail-7 cells were transduced with lentiviruses expressing control or PRL2 shRNA were cultured with or without IL-7 for 2 days. The percentage of apoptotic cells was measured by Annexin V and PI staining (\*p<0.05, n=3). **(d)** Inhibiting of PRL2 activity with a small molecule inhibitor (PRLi) decreases the viability of human T-ALL cell lines in a dosage-dependent manner. **(e)** Treatment of human T-ALL cell lines with PRL2 inhibitor (PRLi) induces apoptosis (\*\*\*p<0.001, n = 3). **(f)** Immunoblot analysis of ERK phosphorylation in human T-ALL cells following DMSO or a small molecule inhibitor (PRLi) treatment. Representative Western blot analysis of indicated proteins is shown.

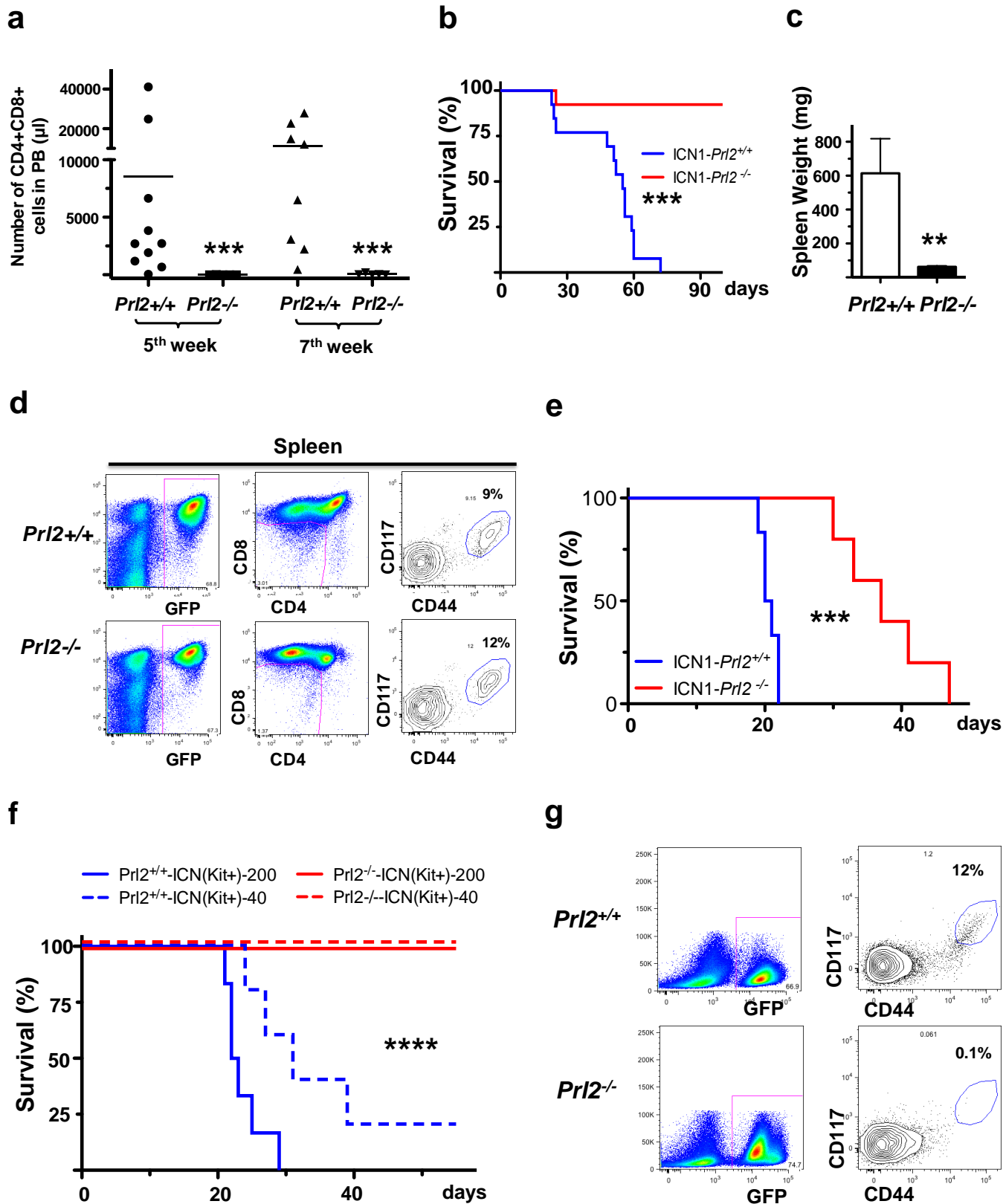
**Figure 2.** PRL2 promotes oncogenic NOTCH1 induced T-ALL *in vivo*. **(a)** Lethally irradiated B6.SJL mice were transplanted with *Prl2*<sup>+/+</sup> or *Prl2*<sup>-/-</sup> progenitor cells expressing NOTCH1-ICN1, and peripheral blood was collected and the frequency of donor-derived (GFP<sup>+</sup>) CD4<sup>+</sup>CD8<sup>+</sup> cells was determined by FACS analysis (n=10, \*\*\*p<0.001). **(b)** Lin<sup>-</sup> bone marrow cells isolated from *Prl2*<sup>+/+</sup> and *Prl2*<sup>-/-</sup> mice were transduced with retroviruses expressing NOTCH1-ICN1 and equivalent number of transduced cells (GFP<sup>+</sup>) were injected into lethally irradiated recipient mice. Kaplan-Meier curve shows the survival of mice transplanted with cells expressing NOTCH1-ICN1 for the period of observation (n=12, \*\*\*p<0.001). **(c)** Average spleen weight in recipient mice repopulated with *Prl2*<sup>+/+</sup> or *Prl2*<sup>-/-</sup> cells expressing NOTCH1-ICN1 (n=10, \*\*p<0.01). **(d)** *Prl2*<sup>+/+</sup> and *Prl2*<sup>-/-</sup> fetal liver cells were transduced with retroviruses expressing NOTCH1-ICN1 and equivalent number of transduced cells (GFP<sup>+</sup>) were injected into lethally irradiated recipient mice. Representative flow cytometry blots showing the frequency of leukemia-initiating cells (Kit<sup>+</sup>CD44<sup>+</sup>CD25<sup>+</sup>) in the spleen of primary recipient mice 4 weeks following

transplantation. (e) 20,000 GFP<sup>+</sup> cells isolated from the spleens of primary recipient mice were injected into lethally irradiated recipient mice. Kaplan-Meier curve shows the survival of mice transplanted with cells expressing NOTCH1-ICN1 for the period of observation (\*\*p<0.001, n=5). (f) 40 or 200 leukemia-initiating cells (Kit<sup>+</sup>CD44<sup>+</sup>CD25<sup>+</sup>) purified from the spleens of primary recipient mice were injected into lethally irradiated recipient mice. Kaplan-Meier curve shows the survival of mice transplanted with cells expressing NOTCH1-ICN1 for the period of observation (\*\*\*\*p<0.0001, n=5). (g) Representative flow cytometry blots showing the frequency of leukemia-initiating cells (Kit<sup>+</sup>CD44<sup>+</sup>CD25<sup>+</sup>) in the spleens of secondary recipient mice repopulated with leukemia-initiating cells (Kit<sup>+</sup>CD44<sup>+</sup>CD25<sup>+</sup>) expressing NOTCH1-ICN1.

**Figure 1**



**Figure 2**



## **Phosphatase PRL2 promotes oncogenic NOTCH1-induced T cell leukemia**

### **Supplemental Methods**

#### **Mice**

Wild type C57BL/6 (CD45.2<sup>+</sup>) and B6.SJL (CD45.1<sup>+</sup>) mice were purchased from the Jackson Laboratories. *Prl2*<sup>+/+</sup> and *Prl2*<sup>-/-</sup> mice were maintained in the Indiana University Animal Facility and kept in Thorensten units with filtered germ-free air. All mice were 10-12 weeks of age at the time of analysis. Both male and female mice were utilized in the experiments. The Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of Medicine approved all experimental procedures.

#### **Human T-ALL cell lines and cord blood cells**

Human T-ALL cell lines, including HPB-ALL, Loucy, CEM, Tail-7, Molt4, and Supt1, were obtained from ATCC. All cell lines were authenticated by SRT profiling and tested for mycoplasma contamination. Normal human cord blood samples were obtained after informed consent following the guidelines of the institutional review board of the Indiana University School of Medicine.

#### **Production of Lentivirus**

Lentiviral shRNA plasmid (pLB) was purchased from Addgene (11619). Oligonucleotides targeting control (Luciferase) and human PRL2 mRNAs were cloned into the pLB plasmid. Oligonucleotide sequences are available upon request. Lentiviral particles were generated by standard method using the third generation packaging system (pMDL, pMD2.G, and pRSV-Rev). Human T-ALL cell lines were infected with high-titer lentiviral suspensions. Twenty-four hours after infection, GFP-positive cells were sorted by FACS. The reduction of PRL2 proteins was determined by immunoblot analysis.

#### **Immunoblotting analysis**

Cells were washed with ice-cold PBS, and lysed on ice for 30 min in lysis buffer (50 mM Tris-HCl, pH

7.4, 150 mM NaCl, 1% Triton X-100, and 10% glycerol) supplemented with a Complete Protease Inhibitor tablet (Roche Applied Science). Cell lysates were cleared by centrifugation at 15,000 rpm for 10 min. The lysate protein concentration was estimated using a BCA protein assay kit (Pierce). The protein samples were boiled with sample buffer, separated by SDS-PAGE, transferred electrophoretically to a nitrocellulose membrane, and immunoblotted with appropriate antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were developed by the enhanced chemiluminescence technique (ECL kit, GE Healthcare). Representative results from at least two independent experiments are shown. Antibodies were purchased from Cell Signaling (ERK, pERK, c-Kit, and Actin) and Abcam (Histone H3).

### **Apoptosis assays**

Human T-ALL cell lines were treated with DMSO or different concentration of PRL inhibitor (PRLi). 24 hours later, cell viability was evaluated by PI/Annexin V staining. Apoptotic cells were defined as PI<sup>-</sup> Annexin V<sup>+</sup>.

### **Production of Retrovirus**

Retroviral particles were produced by transfection of Phoenix E cells with the MSCV-IRES-GFP or MSCV- NOTCH1-ICN1-IRES-GFP plasmids, according to standard protocols. Mouse hematopoietic progenitor cells were transduced on retronectin (Takara)-coated non-tissue culture plates with high-titer retroviral suspensions. Twenty-four hours after infection, GFP-positive cells were sorted by FACS. Transduced cells were then transplanted into lethally irradiated recipient mice. The presence of CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) leukemic cells in the peripheral blood was measured by flow cytometry analysis.

### **Real-time PCR**

For quantitative reverse-transcription polymerase chain reaction (RT-PCR), total RNA was isolated using the RNeasy Plus Micro Kit (QIAGEN), and then subjected to reverse transcription with random hexamers (SuperScript III kit; Invitrogen). Quantitative real-time PCR was performed with SYBR Green (Qiagen) on an ABI PRISM 7500 system. Gene specific primers were designed to flank introns so that products from cDNA could be distinguished from possible genomic DNA contamination. Primer sequences are available upon request.

### **Statistical Analysis**

The animal sample size was based on previous studies evaluating the roles of oncogenic Notch in leukemia and POWER analysis. Using Chi-Square analysis, 5 mice per group will provide 80% POWER in detecting difference with 95% difference. Gehan-Breslow-Wilcoxon test was used for Kaplan-Meier survival curves. The other data were analyzed by paired or unpaired t test using GraphPad Prizm 5. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ; ns, not significant. All experiments were repeated at least once.

### **Supplementary Figure Legends**

**Figure S1.** (a) Tail-7 cells were transduced with lentiviruses expressing control (Sh-Luc) or PRL2 shRNAs (Sh-PRL2-1 and Sh-PRL2-2). The level of PRL2 proteins was determined by western blot analysis. (b) CEM cells were transduced with lentiviruses expressing control or PRL2 shRNA. The proliferation of transduced cells ( $GFP^+$ ) was measured over time (\* $p < 0.05$ ,  $n=3$ ). (c) Molt4 cells were transduced with retroviruses expressing dominant-negative PRL2 (PRL2-CSDA) or GFP. The proliferation of transduced cells ( $GFP^+$ ) was measured over time (\*\* $p < 0.01$ ,  $n=3$ ). (d) PRL2 inhibitor (PRLi) treatment did not affect the viability of human cord blood mononuclear cells ( $p < 0.1$ ,  $n = 3$ ). (e) The Frequency of  $CD34^+$  cells in human cord blood mononuclear cells was determined by flow cytometry analysis. Representative flow cytometry plots were shown. (f) PRL2 inhibitor (PRLi) treatment did not affect the viability of human cord blood  $CD34^+$  cells ( $p < 0.1$ ,  $n = 3$ ).



**Figure S2.** (a) Representative pictures for spleen isolated from recipient mice repopulated with *Prl2*<sup>+/+</sup> or *Prl2*<sup>-/-</sup> cells expressing NOTCH1-ICN1. (b) Representative flow cytometry plots for analyzing myeloid cell, B cell, CD4<sup>+</sup> and CD8<sup>+</sup> T cell engraftment in the surviving recipients. (c) Lin<sup>-</sup>Sca1<sup>+</sup> bone marrow cells isolated from *Prl2*<sup>+/+</sup> and *Prl2*<sup>-/-</sup> mice were transduced with retroviruses expressing NOTCH1-ICN1 or GFP. Transduced cells (GFP<sup>+</sup>) were cultured in liquid medium in the presence of SCF, FLT3 ligand, and IL-7. Cell proliferation was measured at day 11. (d) Lin<sup>-</sup>Sca1<sup>+</sup> bone marrow cells isolated from *Prl2*<sup>+/+</sup> and *Prl2*<sup>-/-</sup> mice were transduced with retroviruses expressing NOTCH1-ICN1 or GFP. Expression of c-Kit in transduced cells (GFP<sup>+</sup>) was determined by western blot analysis at day11 and day17. *Prl2*<sup>-/-</sup> cells (GFP<sup>+</sup>) failed to growth at day17. (e) The level of PRL2 mRNA in splenocytes isolated from recipient mice repopulated with *Prl2*<sup>+/+</sup> cells expressing NOTCH1-ICN1 was determined by quantitative real-time PCR analysis (\*p<0.05, n=3). (f) The level of PRL2 proteins in splenocytes isolated from recipient mice repopulated with *Prl2*<sup>+/+</sup> and *Prl2*<sup>-/-</sup> cells expressing NOTCH1-ICN1 was determined by western blot analysis. Splenocytes from *Prl2*<sup>+/+</sup> mice or recipients repopulated with *Prl2*<sup>+/+</sup> bone marrow cells were used as controls. (g)  $\gamma$ -secretase inhibitor (GSI) treatment decreased the level of PRL2 proteins in some human T-ALL cell lines.

Figure S1

