Notch1 Receptor Regulates AKT Protein Activation Loop (Thr<sup>308</sup>) Dephosphorylation through Modulation of the PP2A Phosphatase in Phosphatase and Tensin Homolog (PTEN)-null T-cell Acute Lymphoblastic Leukemia Cells*<sup>[1]</sup>

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**Background:** PTEN loss promotes resistance to γ-secretase inhibitors by increasing AKT signaling in T-cell acute lymphoblastic leukemia (T-ALL) with mutant activated Notch1.

**Results:** Notch1 inhibition increases AKT phosphorylation and involves the PP2A phosphatase.

**Conclusion:** Notch1 regulates PP2A dephosphorylation of AKT-Thr<sup>308</sup> by impacting association of PP2A with AKT.

**Significance:** Better understanding of regulation of AKT signaling by Notch1 may lead to new therapies for T-ALL.

Notch1 activating mutations occur in more than 50% of T-cell acute lymphoblastic leukemia (T-ALL) cases and increase expression of Notch1 target genes, some of which activate AKT. HES1 transcriptionally silences phosphatase and tensin homolog (PTEN), resulting in AKT activation, which is reversed by Notch1 inhibition with γ-secretase inhibitors (GSIs). Mutational loss of PTEN is frequent in T-ALL and promotes resistance to GSIs due to AKT activation. GSI treatments increased AKT-Thr<sup>308</sup> phosphorylation and signaling in PTEN-deficient, GSI-resistant T-ALL cell lines (Jurkat, CCRF-CEM, and MOLT3), suggesting that Notch1 represses AKT independent of its PTEN transcriptional effects. AKT-Thr<sup>308</sup> phosphorylation and downstream signaling were also increased by knocking down Notch1 in Jurkat (N1KD) cells. This was blocked by treatment with the AKT inhibitor perifosine. The PI3K inhibitor wortmannin and the protein phosphatase type 2A (PP2A) inhibitor okadaic acid both impacted AKT-Thr<sup>308</sup> phosphorylation to a greater extent in nontargeted control than N1KD cells, suggesting decreased dephosphorylation of AKT-Thr<sup>308</sup> by PP2A in the latter. Phosphorylations of AMP-activated protein kinase α (AMPKα)-Thr<sup>172</sup> and p70S6K-Thr<sup>389</sup>, both PP2A substrates, were also increased in both N1KD and GSI-treated cells and responded to okadaic acid treatment. A transcriptional regulatory mechanism was implied because ectopic expression of dominant-negative mastermind-like protein 1 increased and wild-type HES1 decreased phosphorylation of these PP2A targets. This was independent of changes in PP2A subunit levels or in vitro PP2A activity, but was accompanied by decreased association of PP2A with AKT in N1KD cells. These results suggest that Notch1 can regulate PP2A dephosphorylation of critical cellular regulators including AKT, AMPKα, and p70S6K.

Pediatric acute lymphoblastic leukemia (ALL)<sup>3</sup> treatments have improved for B-precursor ALL as most patients today experience an excellent prognosis with ~90% 5-year event-free survivals (1). However, T-cell ALL (T-ALL), which accounts for 10–15% of cases, is associated with 5-year event-free survivals of ~70–75% with current intensive therapies (2). Relapsed T-ALL and early T-cell precursor ALL are refractory to treatment (3, 4). T-ALL is accompanied by fewer unique chromosomal abnormalities than B-precursor ALL, although frequent but favorable translocations have been identified with the T-cell receptor involving TAL1, HOX11/TLX1, and Notch1 (5). Notch1 is of particular interest in T-ALL as Notch1 activating mutations were reported in greater than 50% of cases (6), including early T-cell precursor ALL (7), and were associated with better treatment responses and reduced rates of relapse (8).

Notch1 is a heterodimeric receptor with N-terminal extracellular (NEC) and transmembrane domains, and a C-terminal intracellular transcriptional transactivation domain, noncovalently associated through a heterodimerization (HD) domain (9). Binding DSL (Delta-Serrate-Lag1) ligands to the NEC EGF-

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**The abbreviations used are:** ALL, acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia; AMPK, AMP-activated protein kinase; cpd-E, compound-E; GSI, γ-secretase inhibitor; GSK3, glycosynthesis kinase 3; HES1, hairy and enhancer of split-1; ICN1, intracellular Notch1; IP, immunoprecipitation; MAML1, mastermind-like protein 1; mTOR, mammalian target of rapamycin; N1KD, Notch1 knockdown; NTC, nontargeted control; OA, okadaic acid; PHLP, pleckstrin homology domain and leucine-rich repeat protein phosphatases 1 and 2; PP2A, protein phosphatase type 2A; PTEN, phosphatase and tensin homolog; HD domain, heterodimerization domain; LNR, Lin12/Notch1 repeat; p70S6K, p70S6 kinase; DMSO, dimethyl sulfoxide; DAPT, N-(3,5-difluorophenacetyl)-L-alanyl-S-phenylglycine t-butyl ester.

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like repeats activates Notch1 by promoting proteolytic cleavages, initially at the S2-site by an ADAM (a disintegrin and metalloprotease) protease followed by cleavage at the S3-site by the γ-secretase complex, releasing intracellular Notch1 (ICN1) (9). In the nucleus, ICN1 forms a transcriptional activation complex by recruiting CSL (CBF1/Su(H)/Lag-1), which provides an interface to associate with mastermind-like protein 1 (MAML1) activator proteins, displacing co-repressors and recruiting co-activators (9). Genes directly activated by ICN1 include PRE-Tα (10), HES1 (11), DELTEX1 (12), c-MYC (13), IGF1R (14), and IL7R (15).

The HD and PEST domains are “hotspots” for activating Notch1 mutations in T-ALL (6). The Lin12/Notch1 repeats (LNRs) aid in stabilizing the HD domain, preventing ligand-independent S2 cleavage (9). Mutations within the HD domain result in HD/LNR destabilization, insertions that increase the distance between the HD and LNR repeats decrease LNR masking of the S2 cleavage site, and Notch1 extracellular juxtamembrane expansion (JME) mutations increase the distance of the HD/LNR from the membrane, all resulting in ligand-independent activation of Notch1 (16). Mutations within the PEST domain increase ICN1 stability. A similar effect results from mutations in the E3-ubiquitin ligase, FBW7 (17). Thus, both Notch1 and FBW7 mutations increase Notch1 signaling activity in T-ALL (6, 17, 18).

AKT (PKB) regulates cell proliferation, growth, survival, and metabolism (19). Phosphatidylinositol 3-kinase (PI3K) phosphorylates phosphatidylinositol (4,5)-biphosphate to phosphatidylinositol (3,4,5)-trisphosphate and recruits AKT to the membrane, where it is activated through phosphorylation of its activation loop (Thr308) and hydrophobic motif (Ser473) by phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin (mTOR) complex 2 (mTOR2), respectively (19). Protein phosphatase 2A (PP2A) and pleckstrin homology domain leucine-rich repeat protein phosphatases (PHLPP) 1 and 2 are major Ser/Thr phosphatases that dephosphorylate AKT (20). PP2A has been reported to dephosphorylate AKT-Thr308 (21) and AKT-Ser473 (22), whereas PHLPP dephosphorylates AKT-Ser473 (23). Although PHLPP primarily regulates AKT, PP2A dephosphorylates other phospho-proteins in addition to AKT (24), including but not limited to c-Myc (25), p70S6K (26), and AMP-activated protein kinase (AMPK) (27).

Notch1 has been reported to regulate AKT in T-ALL (14, 28–30). Regulation of AKT was described as indirect, involving HES1 transcriptional repression of phosphatase and tensin homolog (PTEN) (30), a phosphatidylinositol (3,4,5)-triphosphate phosphatase that antagonizes AKT activation (19). Notch1 mutations result in ligand-independent activation and elevated HES1, with decreased PTEN levels and sustained AKT activity (30). γ-Secretase inhibitor (GSI) treatments restore PTEN levels, resulting in decreased AKT phosphorylation, with loss of cell proliferation and increased chemotherapy-induced apoptosis, which is abrogated in cells that have lost PTEN (30, 31). However, mutation and inactivation of PTEN are common events in cancer (19), including T-ALL (30, 32), and are associated with GSI resistance in T-ALL (30). This may reflect the inability of GSIs to block chronic AKT activation in cells that lack PTEN (30). Although Notch1 may regulate AKT independent of PTEN (14), this has not been systematically studied.

In this study, we explore the regulation of AKT in PTEN-null T-ALL cells. Our results establish that suppression of Notch1 by GSI treatment or shRNA knockdown of Notch1 increases AKT phosphorylation at Thr308 and Ser473, resulting in activation of downstream effectors. This appears to reflect decreased dephosphorylation of AKT by PP2A, at least in part mediated by HES1. An analogous effect on phosphorylation of AMPKα-Thr172 and p70S6K-Thr389, both substrates of PP2A, was also observed (26, 27), suggesting a role for Notch1 in regulating PP2A substrate specificities. Notch1 knockdown significantly decreased the association of AKT with PP2A, providing an explanation for the observed increased phosphorylation of AKT-Thr308. To our knowledge, our findings that Notch1 regulates AKT signaling at the level of PP2A and that Notch1 regulates AMPKα-Thr172 phosphorylation are completely unprecedented.

EXPERIMENTAL PROCEDURES

Antibodies, Expression Constructs, and Drugs—A list of the antibodies used in these studies is provided in supplemental Table 1S. A pFLAG-CMV-2 expression vector harboring a dominant-negative MAML1 (MAML 1–302) and an empty pFLAG-CMV-2 vector were provided by Dr. L. Wu (University of Florida) (33). The full-length human HES1 coding sequence (237–1390 nucleotides, GenBank accession number: NM_005524.3) in the pGEM-T Easy vector was provided by Dr. R. Kageyama (Kyoto University, Japan) (34). The EcoRI-HES1-Spel-EcoRI fragment was removed from the pGEM-T Easy vector with EcoRI restriction enzyme and ligated in-frame into the pcDNA3 mammalian expression vector (Invitrogen) EcoRI site and under control of the CMV promoter. Okadaic acid (OA) and wortmannin were purchased from EMD Millipore. Compound-E (cpd-E) was from Enzo Life Sciences, and DAPT (GSI-IX) (N-(N-(3,5-difluorophenacetyl)-l-alanyl)-S-phenylglycine t-buty1 ester) and perifosine were purchased from Selleck Chemicals. With the exception of perifosine, drugs were dissolved in dimethyl sulfoxide (DMSO). Perifosine was dissolved in sterile deionized water.

Cell Lines—GSI-resistant T-ALL cell lines, Jurkat, MOLT3, and CCRF-CEM, were purchased from the American Type Culture Collection (ATCC). Cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Scientific), penicillin (100 units/ml final)/streptomycin (100 μg/ml final), and 2 mM l-glutamine (Invitrogen). Cells were grown in a 37 °C humidified incubator in the presence of 5% CO2.

For certain experiments, cells were “serum-starved” by maintenance (at 0.5 × 106 cell/ml) in the presence of reduced serum (0.2% FBS) for 20–24 h. Afterward, equal quantities of viable cells (determined by trypan blue dye exclusion and manual counting using a hemocytometer) were pelleted (530 × g, 5 min at 4 °C) and then resuspended into fresh medium containing 10% FBS to a density of 0.5 × 106 cell/ml for 30 min at 37 °C prior to experiment.

For transient transfections, 1 × 107 cells were mixed with 40 μg of plasmid DNA and allowed to incubate at room tempera-
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ture for 15 min in electroporation cuvettes (0.4-cm gap width, Bio-Rad) prior to electroporation with the Gene Pulser Xcell system (Bio-Rad) set at 250 V, 1000 microfarads, and infinite resistance. Afterward, the cells were allowed to rest at room temperature for another 15 min before suspension into fresh medium for 48 h. Puromycin (250 ng/ml) (InvivoGen) was added (as needed) as a selection marker.

Notch1 Knockdown in Jurkat Cells by shRNA Lentiviral Transduction—Notch1 (NM_017617.3) targeted shRNA oligonucleotides (5’-CACCACAGATCAATGAGTTCAGTG CAGTGCCGAAGCCTGCACTGAATCA TTGATCTTGTTG-3’ (upper) and 5’-AAAAACAGATCAATGAGTC CAGTGCCGAAGCCTGCACTGAATCA TTGATCTTGTTG-3’ (lower)) directed against the 1520–1548 region of exon 9 (corresponding to LNR2-LNR3 of the extracellular domain of Notch1) were designed using Gene Link™ RNAi explorer and synthesized by Invitrogen (the underlined sequence is the shRNA loop region). The shRNA was annealed, inserted into the pENTR™/H1/TO cloning vector, and transformed into One Shot TOP10 chemically competent Escherichia coli cells (Invitrogen). Notch1-targeted shRNA was packaged in MISSION™ pLKO.1-puro lentiviral vector by Sigma. Nontargeted control (NTC) shRNA lentiviral particles (Sigma, catalog number: SHC002V) were also prepared. Jurkat cells (2 × 10⁵ cells) were treated with Notch1-targeted or NTC shRNA lentiviral particles (10⁵ transducing units/ml) at a multiplicity of infection of 0.1 and 4 × 10⁶ Polybrene in a 24-well plate format for 24 h. Afterward, viral particles were removed by centrifugation (530 × g, 5 min at 4 °C). The Notch1 knockdown (N1KD) clones were isolated from soft agar and selected using 250 ng/ml puromycin. The N1KD clones were screened by real-time PCR and Western blotting (below). The NTC and N1KD clones used for our experiments (designated N1KD4 and N1KD7) were maintained as above, except that puromycin (250 ng/ml) was included.

Western Blotting—Cells were lysed by sonication in 10 mM Tris/HCl (pH 7.5), 0.5% SDS, 1 × protease inhibitor mixture (Roche Applied Science), and 1 × phosphatase inhibitor tablets (PhosSTOP) (Roche Applied Science). Lysates were cleared by centrifugation (14,000 × g, 4 °C), and total protein concentrations were determined using the DC protein assay kit according to manufacturer (Bio-Rad). Lysates (40 µg) were mixed with sample loading buffer containing 2-mercaptoethanol and heated prior to loading the samples onto 10% SDS-polyacrylamide gels using the Laemmli buffer system (35). Samples were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Pierce) and blocked using Odyssey blocking buffer (Li-COR), diluted 1:1 with 1 × phosphate-buffered saline (PBS) (blocking buffer). The blots were probed overnight at 4 °C with primary antibodies (supplemental Table 1S) diluted in blocking buffer supplemented with Tween 20 (0.1%). The blots were washed with 1 × PBS prior to incubating with IR Dye®800 anti-rabbit or anti-mouse secondary antibodies (Li-COR), each diluted to 1:10,000 in blocking buffer supplemented with Tween 20 (0.05%) and SDS (0.02%). A tertiary detection was required to visualize ICN1. For this, blots were probed as above with an ICN1 antibody (Cleaved Notch1 (Val1744) (supplemental Table 1S) diluted to 1:250 and then probed for 1 h at room temperature with goat-anti-rabbit secondary antibody followed by an IR Dye®800 anti-goat tertiary antibody (Rockland Immunochemicals) diluted to 1:1000 in blocking buffer supplemented with TWEEN 20 (0.05%) and SDS (0.02%). All washes used 1 × PBS supplemented with TWEEN 20 (0.1%). Blots were rinsed with 1 × PBS prior to imaging with an Odyssey infrared imaging system (LI-COR). Densitometry of the raw band intensities was carried out with the Odyssey (V3.0) software, according to the manufacturer’s instructions. Densitometry values were corrected for background and normalized. To remove bound antibodies for successive detection of multiple proteins, blots were stripped with 25 mM glycine and 69.3 mM SDS (pH 2.0) buffer at least twice for 15 min prior to reprobing.

Real-time PCR—RNA was prepared from cells with TRIzol® reagent (Invitrogen). Total RNA (2 µg) was reverse-transcribed into cDNA with random hexamer primers and MuLV reverse transcriptase (Applied Biosystems) for 1 h at 42 °C in a PCR machine. Reactions were terminated by heating at 95 °C for 10 min. cDNA was purified using the QiAquick PCR Purification kit according to the manufacturer (Qiagen) and eluted into PCR-grade deionized water. TaqMan probes for relative levels of transcripts for the individual PP2A subunits, PPP2CA, PPP2R2A, PPP2RB5B, and PPP2R5C, were purchased and used as described by the manufacturer (Applied Biosystems). TaqMan assays were run on a LightCycler 480 real-time PCR machine (Roche Applied Science). Levels of HES1 and DELTEX1 transcripts were measured and normalized to GAPDH by real-time PCR using a LightCycler real-time PCR machine (Roche Applied Science) and a LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Applied Science) (18). Relative transcript levels were determined using the 2⁻ΔΔCt method (36).

Cell Proliferation and Cell Cycle Analysis—NTC and N1KD cells were seeded at 7.5 × 10⁴ cells/ml in medium supplemented with puromycin (250 ng/ml) and cultured for 96 h, sampling every 24 h for manual cell counting using trypan blue dye exclusion and a hemocytometer. For cell cycle analysis, cells (0.5–1 × 10⁶) were resuspended into ice-cold 1 × PBS (1 ml) and fixed by adding an equal volume of ice-cold absolute ethanol dropwise while vortexing. Samples were stored at 4 °C. The cells were pelleted by centrifugation (530 × g for 5 min at 4 °C) prior to resuspending into 50 µg/ml propidium iodide and 100 µg/ml RNAse type I-A (Sigma-Aldrich) in 1 × PBS. Samples were incubated at room temperature in the dark for a minimum of 20 min prior to analyzing by flow cytometry using a BD FACSauto 4™ II flow cytometer operated with BD FACSDiva™ software (v6.0) (BD Biosciences). A minimum of 1 × 10⁶ events was collected. All data were analyzed with FlowJo (v7.6.1) software (Tree Star, Inc.), using cell cycle analysis with the Watson Pragmatic model.

PP2A Activity Assay—NTC and N1KD7 cells (1.25 × 10⁵ cells/ml) were cultured for 48 h. To harvest the cultures, cells were washed with 1 × Tris-buffered saline (1 × TBS), pH 7.2 (Pierce), and lysed in PP2A activity assay buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 1× protease inhibitor mixture (Roche Applied Science)) (37) for 1 h at 4 °C. Lysates were cleared by centrifugation, and total
protein concentrations were measured with the DC protein assay (Bio-Rad). Lysates (200 μg) were incubated with PP2A C subunit (clone 1D6) antibody (8 μg) (supplemental Table 1S). The PP2A activity assay was performed using the PP2A immunoprecipitation phosphatase assay kit and a synthetic phosphopeptide according to the manufacturer’s instructions (Millipore). Immunoprecipitated PP2A was divided into two fractions and treated with either OA (100 nM; sufficient to selectively inhibit PP2A in vitro (38)) or DMSO for 10 min at 30 °C prior to the addition of the phospho-peptide substrate for an additional 10 min. Malachite green reagent was used to detect free phosphate liberated from the phospho-peptide by PP2A using a microtiter plate reader set at 650 nm.

Assay of PP2A-AKT Association by Co-immunoprecipitation Assays—NTC and both N1KD sublines (1.25 × 10^6 cells/ml) were cultured for 48 h and then washed with 1× TBS prior to lysing in co-immunoprecipitation (co-IP) buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1× protease and 1× phosphatase inhibitor mixture (Roche Applied Science)) (39) for 2 h. All steps were carried out on ice or at 4 °C. Lysates were cleared by centrifugation, and total protein concentrations were measured and adjusted using the DC protein assay (Bio-Rad). Lysates were diluted to 1 μg/μl of protein with co-IP buffer and cleared with protein-A-agarose beads (Roche Applied Science) for 1 h. The cleared supernatants (250 μg) were transferred to new tubes and treated with PP2A C subunit (clone 1D6) mouse monoclonal antibody at a titer of 1:25 (Millipore) (supplemental Table 1S) for 16–20 h. For a control (“mock”) co-IP, normal mouse IgG (Millipore) was added. The antibody-antigen complexes were bound to protein-A-agarose beads (Roche Applied Science) over 4 h at 4 °C while mixing. The beads were washed (four times, 10 min each wash) with 500 μl of co-IP buffer. Laemmli sample loading buffer was added to the immunoprecipitates, followed by heating (95–100 °C) to release bound proteins. The supernatants were analyzed by Western blotting on 10% SDS-PAGE gels run at 200 V for 65 min to resolve proteins running near the IgG heavy chain. Blots were probed with assorted antibodies (supplemental Table 1S) at a 1:500 dilution for 72 h at 4 °C. Immunoreactive proteins were detected with the Odyssey infrared imaging system and quantified by densitometry with the Odyssey (V3.0) software.

Statistics—For experiments where statistical analysis was applied, three independent biological replicates were analyzed for statistical significance using the Student’s t test. Any p value equal to or less than 0.05 (95% confidence interval) was considered to be statistically significant. Data were plotted and analyzed with GraphPad Prism 4 software (GraphPad Software, Inc.).

RESULTS

Notch1 Inhibition with Compound-E or shRNA Targeted Knockdown of Notch1 Increases AKT Phosphorylation and Signaling in PTEN-null T-ALL Cell Lines—Jurkat cells are PTEN-null (32) and harbor a juxtamembrane expansion activating mutation in the Notch1 receptor, which is sensitive to GSI treatment (16). Treating Jurkat cells with the GSI cpd-E (0–2 μM for 72 h) decreased ICN1 levels and led to a dose-dependent increase in phosphorylation of AKT at both Thr^{308} and Ser^{473} (Fig. 1A). The effect was more pronounced for AKT-Thr^{308} and correlated with phosphorylation of GSK3α/β, a direct AKT substrate (40). However, PI3Kα levels were unaffected by cpd-E (Fig. 1A). Analogous effects on AKT signaling were obtained upon treatment of Jurkat cells with another GSI, DAPT (GSI-IX) (41) (Fig. 1B). To investigate the generality of this response to GSI treatment, two additional GSI-resistant T-ALL cell lines, CCRF-CEM and MOLT3, characterized as PTEN-null and harboring mutant active Notch1 (17, 30) (CCRF-CEM cells also have a mutant FBW7 (17)), were treated with 2 μM cpd-E for 72 h. AKT-Thr^{308} and GSK3α/β phosphorylations were preferentially increased by cpd-E in both cell lines, whereas the effect on AKT-Ser^{473} phosphorylation was variable despite constant PI3K levels (Fig. 1C). These results suggest the existence of a generalized mechanism whereby Notch1 inhibition activates AKT signaling in GSI-resistant T-ALL cells.

GSIs are promiscuous and can affect the activity of at least 60 other type 1 transmembrane proteins including all the mammalian Notch receptors (Notch1–4) (42, 43). To confirm a role of Notch1 in the increased AKT-308 phosphorylation, Jurkat cells were transduced with a lentivirus expressing shRNA directed toward Notch1 to knockdown the protein. Notch1 protein levels were profoundly decreased in two N1KD clones (designated N1KD4 and N1KD7) (Fig. 2A). Functional knockdown of ICN1 was confirmed by measuring HES1 and DELTEX1 transcripts as both N1KD4 and N1KD7 cells showed markedly decreased transcript levels for HES1 (0.098 ± 0.015 for N1KD4 (p < 0.0001) cells and 0.134 ± 0.042 for N1KD7 (p < 0.0001) cells, where NTC cells are assigned a value of 1) and DELTEX1 (0.206 ± 0.100 for N1KD4 (p = 0.0014) cells and 0.179 ± 0.066 for N1KD7 (p = 0.0002) cells) (Fig. 2A). Thus, the extent of Notch1 knockdown in the N1KD4 and N1KD7 cells was clearly sufficient to impair Notch1 signaling.

Similar to the results for wild-type Jurkat cells treated with cpd-E (Fig. 1), both N1KD4 and N1KD7 cells exhibited elevated AKT phosphorylation as compared with the NTC cells that was more pronounced for Thr^{308} than Ser^{473} and was accompanied by increased GSK3α/β phosphorylation (Fig. 2A). Again, these changes were independent of PI3Kα levels. These results further suggest a unique role for Notch1 in activating AKT signaling independent of PTEN. Because both N1KD4 and N1KD7 cells similarly affect AKT signaling, we used the N1KD7 cells for the majority of our studies, with comparisons to GSI-treated cells and N1KD4 cells, as appropriate.

To further confirm the impact of loss of Notch1 activity on AKT signaling, N1KD7 and NTC cells were treated for 24 h with the specific AKT inhibitor, perifosine (20 μM), previously shown to block AKT phosphorylation and signaling in Jurkat cells (44). In the N1KD7 and NTC cell lines, perifosine potently inhibited AKT phosphorylation and also decreased phosphorylation of FOXO1 and GSK3α/β, both direct AKT substrates (40, 45) (Fig. 2B).

Notch1 Has Minimal Effects on Cell Proliferation and Cycle—We investigated whether the increased AKT signaling in N1KD7 cells as compared with NTC cells was associated with changes in cell proliferation or cell cycle profile. Proliferation rates (21.29 ± 1.53) and 21.74 ± 3.67 h for N1KD7 and NTC,
respectively) (Fig. 2C) and cell cycle profiles during log-phase growth (72 h) were essentially unchanged (Fig. 2D). These results confirm that differences in rates of cell proliferation do not account for the disparate patterns of AKT signaling between NTC and N1KD7 cells. Further, these results are consistent with the reported nominal effects of GSIs on proliferation rates and the cell cycle profile of Jurkat cells (46).

**Elevated AKT-Thr308 Phosphorylation in N1KD7 Cells Reflects Decreased Dephosphorylation by PP2A Phosphatase**—The extent of AKT phosphorylation is a net result of rates of phosphorylation in response to PI3K and dephosphorylation by the Ser/Thr phosphatases PP2A and PHLPP (20). The apparent disconnect between levels of PI3K and increased AKT-Thr308 phosphorylation (Fig. 2A) in N1KD7 cells strongly implied that this response was independent of PI3K. To further assess the role of PI3K in AKT phosphorylation in response to Notch1 inhibition, we treated NTC and N1KD7 cells with wortmannin, a specific inhibitor of PI3K. To examine the impact of changes in PP2A on AKT-Thr308 phosphorylation, we treated NTC and N1KD7 cells with OA. Changes in turnover of phosphorylated AKT-Thr308 over time in NTC and N1KD7 cells were monitored following inhibitor treatments.

If differences in AKT phosphorylation in response to PI3K activation were causal, we reasoned that treatment with high concentrations of wortmannin should decrease phospho-Thr308 to the same extent in NTC and N1KD7 cells. However, if these were independent of PI3K, differences in rates of phospho-Thr308 turnover between NTC and N1KD7 cells would be expected in the continuous presence of wortmannin. Because steady-state AKT phosphorylation in NTC cultures was initially very low (Fig. 2), for the wortmannin experiments, both NTC and N1KD7 cells were serum-starved (20–24 h) and then restimulated with serum for 30 min. This resulted in comparable AKT-Thr308 phosphorylation at 0 h (Fig. 3A, lane 2). Cells were treated with wortmannin (200 nm) over 20 min, and the decay rates of phospho-Thr308, Ser473 were followed on Western blots. Wortmannin treatment had little impact on phospho-Ser473, but was accompanied by decreased phospho-Thr308 levels, albeit to substantially different extents for the NTC and N1KD7 cells (Fig. 3A). The half-lives of phospho-Thr308 turnover differed by 5-fold between the NTC and N1KD7 cells (NTC > N1KD7) (Fig. 3A). This implied a primary effect at the level of Thr308 dephosphorylation. PHLPP and PP2A are the major phosphatases that regulate AKT dephosphorylation. Although PHLPP primarily dephosphorylates AKT-Ser473 and PP2A principally dephosphorylates AKT-Thr308, specificity for AKT-Thr308 dephosphorylation is not absolute (22, 47). To discriminate between these mechanisms, NTC and N1KD7 cells were treated with OA, which selectively inhibits PP2A but does not affect PP2C-type phosphatases such as PHLPP (38, 47). Therefore, any effect of OA on net AKT-Thr308 phosphorylation would implicate PP2A, whereas a lack of an effect would strongly suggest a role for PHLPP. If there were differences in capacities for dephospho-Thr308.
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**FIGURE 2.** Notch1 shRNA knockdown in Jurkat cells recapitulates the changes in AKT phosphorylation and signaling observed in GSI-treated cells. A, NTC, N1KD4, and N1KD7 cells (each 1.25 × 10^6 cells/ml) were grown for 48 h and analyzed by Western blotting (left panel). Representative data are shown from experiments repeated at least in triplicate. N1KD4 and N1KD7 cells were assayed for HES1 and DELTEX1 transcript changes by real-time PCR (right panel). Results for three replicate experiments (presented as mean values ± standard errors) are shown for N1KD4 cells (gray bars) (HES1, 0.098 (± 0.015) relative units (p < 0.0001); and DELTEX1, 0.206 (± 0.100) relative units (p = 0.0014)) and N1KD7 cells (black bars) (HES1, 0.134 (± 0.042) relative units (p < 0.0001); and DELTEX1, 0.179 (± 0.066) relative units (p = 0.0002)), relative to results for NTC cells (white bars) (arbitrarily set to a value of 1). p values equal to or less than 0.05 (95% confidence interval) were considered as statistically significant (denoted by asterisks). B, the NTC and N1KD7 cells were treated with 0 or 20 μM perifosine for 24 h, and AKT, FOXO1, and GSK3α/β phosphorylations were followed by Western blotting (antibodies are summarized in supplemental Table 1S). In panels A and B, F indicates the phosphorylated protein forms. C, NTC (circles) and N1KD7 (squares) cells (7.5 × 10^6 cells/ml) were outgrown in medium for 96 h, and the doubling times were calculated from triplicate manual counting with trypan blue dye exclusion every 24 h using a hemocytometer. Doubling times for the NTC (21.74 ± 3.67 h) and N1KD7 (21.29 ± 1.53 h) cells were determined and are expressed as the mean values ± standard errors from three independent experiments. D, NTC and N1KD7 cells from the 72-h time point in panel C were analyzed for cell cycle distributions by measuring DNA contents with propidium iodide using flow cytometry and FlowJo (v7.6.1) analysis software (Tree Star, Inc.). The calculated percentages were as follows: G0/G1, fraction, 52.58 (± 6.938) % for NTC and 45.59 (± 4.307) % for N1KD7 cells; S, 30.11 (± 4.746) % for NTC and 33.34 (± 2.876) % for N1KD7 cells; and G2/M, 17.31 (± 5.859) % for NTC and 21.08 (± 3.472) % for N1KD7 cells (data expressed as mean values ± standard errors from three independent experiments).

Phosphorylation of Thr^308 by PP2A between NTC and N1KD7, OA should impact NTC cells to a greater extent than the N1KD7 cells.

These possibilities were tested by treating the NTC and N1KD7 cells with OA (1 μM) for 30 min, and levels of AKT-Thr^308 and -Ser^473 phosphorylation were examined by Western blotting. OA substantially and differentially affected phospho-Thr^308 levels in the NTC and N1KD7 cells (Fig. 3B). In NTC cells treated with OA, phospho-Thr^308 levels rose dramatically (~13.6-fold) over 30 min, whereas OA had a much reduced impact on Thr^308 phosphorylation in the N1KD7 cells (~1.6-fold) over this window. Phospho-Ser^473 was largely unaffected. A qualitatively similar result was obtained upon treating NTC and N1KD7 cells with a range of OA concentrations (0–25 nM) over 48 h (Fig. 3C) (38, 48). Under these conditions, AKT-Thr^308 phosphorylation increased to a greater extent in NTC cells (~15.7-fold) than in the N1KD7 cells (~4.4-fold). Analogous results were obtained with wild-type Jurkat cells treated with OA and cpd-E (data not shown). Collectively, these results suggest that the substantially increased phospho-AKT-Thr^308 and AKT signaling accompanying loss of ICN1 in N1KD7 cells or upon inhibition of Notch1 with cpd-E is due to decreased AKT dephosphorylation by PP2A rather than to increased AKT phosphorylation in response to PI3K activation.

Loss of ICN1 in N1KD7 Cells Results in Increased Phosphorylation of Multiple PP2A Intracellular Targets—Our results showed that loss of ICN1 in N1KD7 cells or in wild-type Jurkat cells treated with cpd-E results in increased net phosphorylation of AKT-Thr^308 with consequent effects on downstream signaling. This appears to involve the major Ser/Thr phosphatase PP2A such that inhibiting or knocking down Notch1 resulted in impaired PP2A dephosphorylation of phospho-AKT-Thr^308.

PP2A is a heterotrimeric protein complex consisting of a structural subunit A, a catalytic subunit C, and a diverse repertoire of interchangeably regulatory B subunits that determine specificity of PP2A for its various cellular targets (24). Regulatory subunits B55α, B56β, and B56γ are important for PP2A to dephosphorylate AKT (21, 22, 49). Likewise, p70S6K (Thr^389) is a PP2A-B56γ substrate (26), whereas PP2A activity toward c-Myc (Ser^62) is determined by the B56α subunit (25). AMPKα (Thr^177) is dephosphorylated by PP2A (27); however, the requisite B subunit is not certain. Evidence for dephosphorylation of phospho-AKT-Ser^473 by PP2A is conflicting and might...
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A

- AKT
- NTC
- N1KD7
- Time (min): 0 5 10 15 20
- P-AKT-T308
- P-AKT-S473
- AKT

B

- OA (1 μM)
- NTC
- N1KD7
- Time (min): 0 5 10 15 30
- P-AKT-T308
- P-AKT-S473
- AKT

C

- OA (1 nM)
- NTC
- N1KD7
- Intensity of Phospho-AKT (T308)

**FIGURE 3. Increased phospho-AKT-Thr308 levels in the N1KD7 cells are the result of decreased PP2A function and not changes in PI3K.** A, NTC (left panel) and N1KD7 cells were serum-starved for 20–24 h (labeled SS, lane 1) and then restimulated with serum (noted with arrow between lanes 1 and 2) for 30 min (see “Experimental Procedures”) and analyzed by Western blotting and densitometry. An aliquot was taken immediately prior (labeled 0 min, lane 1) to the addition of 200 nM wortmannin to the cells for 5, 10, 15, and 20 min (lanes 3–6). The phospho-AKT-Thr308 levels (measured by densitometry) at 0 min were arbitrarily set to 100%, and the rate of decay was plotted (right panel) for NTC (circles) and N1KD7 (squares) cells. Half-lives for phospho-AKT-Thr308 in the NTC (∼3 min) and N1KD7 (∼15 min) cells were estimated from the times at which the signals decreased to 50% of their initial levels. B, a sample was taken immediately prior (labeled 0 min, lane 1) to the treating the NTC and N1KD7 cells with 1 μM OA for 5, 10, and 30 min (lanes 2–4). Samples were analyzed by Western blotting. C, NTC (lanes 1–5) and N1KD7 (lanes 6–10) cells (1.25 × 10^5 cells/ml) were treated with 0, 3.125, 6.25, 12.5, and 25 nM OA for 48 h and analyzed by Western blotting. For panels B and C, OA-induced fold increases in AKT-Thr308 phosphorylation (measured by densitometry) are plotted on semilog plots for both NTC (circles) and N1KD7 cells (squares) (shown below respective Western blots). Antibodies for Western blotting are summarized in supplemental Table 1S. Representative data are shown from experiments repeated at least in triplicate. In all panels, P indicates the phosphorylated forms.

reflect B subunit specificity (21, 22), although phosphorylation of AKT-Ser473 was largely unaffected by OA in our experiments (Fig. 3, B and C). If impaired PP2A dephosphorylation is indeed responsible for the substantially elevated phospho-AKT-Thr308 in N1KD cells or in cells treated with cpd-E, this should be accompanied by parallel effects on other phosphorylated PP2A substrates as well.

Accordingly, phosphorylation of assorted PP2A substrates was examined in the NTC and N1KD7 cells (Fig. 4A). Again, AKT phosphorylation was dramatically increased for Thr308 and, to a lesser extent, Ser473 in N1KD7 cells, as compared with NTC cells. Phospho-AMPKα-Thr172 and phospho-p70S6K-Thr389 levels were also substantially elevated in the N1KD7 cells, whereas phospho-c-Myc-Ser62 levels were unchanged. Similar results were obtained with Jurkat cells treated with cpd-E, with the exception of c-Myc, which was decreased (Fig. 4B). As expected, OA (12.5 nM) treatment for 48 h markedly increased phosphorylation of AKT-Thr308, AMPKα-Thr172, and p70S6K-Thr389 in the NTC cells (Fig. 4A). Detectable, albeit much smaller, relative increases in AKT-Thr308 and AMPKα-Thr172 phosphorylation were seen in the N1KD7 cells treated with OA. No increase was detected in phospho-p70S6K-Thr389 in N1KD7 cells treated with OA. Phosphorylation of AKT-Ser473 was unaffected by OA in both NTC and N1KD7 cells. The decreased phosphorylation of c-Myc-Ser62 in the presence of OA may reflect the dramatically reduced c-Myc levels reported to be induced by prolonged OA treatments (50).

These results establish that inhibition of Notch1 by shRNA knockdown or treatment with cpd-E increases phosphorylation of multiple PP2A substrates including AKT-Thr308, AMPKα-Thr172, and p70S6K-Thr389. As OA did not affect AKT-Ser473 phosphorylation, it seems likely that its regulation by Notch1 does not directly involve PP2A. p70S6K lies downstream from mTOR1 and could be directly regulated by AKT, AMPK, and Notch1 (19, 46), although our OA inhibition results (Fig. 4A) suggest that p70S6K may also be regulated by PP2A.

**Effects of Notch1 on PP2A Are Mediated by MAML1 and HES1—**Notch1 activates genes via generation of ICN1 and is MAML1-dependent (9, 33). To determine whether Notch1 impacts phosphorylation of diverse PP2A substrates through a classic transcriptional mechanism, we transfected a dominant-negative MAML1 construct (33) into wild-type Jurkat cells. Analogous to the results with N1KD7 cells or Jurkat cells treated with cpd-E, dominant-negative MAML1 by itself increased phosphorylation of AKT-Thr308, AMPKα-Thr172, and p70S6K-Thr389 (Fig. 4C). Thus, Notch1 regulates phosphorylation of these PP2A substrates, most likely through a transcriptional mechanism, although it is unclear whether this involves a direct effect of ICN1 on gene targets likely to impact
PP2A dephosphorylation or whether this is mediated by a downstream Notch1 gene target such as HES1.

HES1 functions as a transcriptional repressor (51). HES1 was reported to repress transcription of the PTEN gene, leading to increased AKT signaling in cells with wild-type PTEN (30). This suggested a causal role for HES1 in the indirect regulation of AKT by Notch1. Further, HES1 contributes to establishment and maintenance of a quiescent phenotype (52), characterized by elevated HES1 and decreased AKT phosphorylation (53). Again, this suggests that HES1 is involved in AKT regulation.

We sought to test the concept of whether the effects of Notch1 on steady-state AKT-Thr308 phosphorylation in PTEN-null Jurkat cells could be mediated at least in part by HES1. If HES1 was involved, we reasoned that by ectopically expressing HES1 in N1KD7 cells, which have very low HES1 expression, HES1 would be involved, we reasoned that by ectopically expressing HES1 in N1KD7 cells, which have very low HES1 expression, this would decrease net AKT-Thr308 phosphorylation levels. When HES1 was ectopically expressed in N1KD7 cells, phosphorylation levels of AKT-Thr308, AMPKα-Thr172, and p70S6K-Thr389 were all dramatically decreased (Fig. 4D). Again, there was no effect of HES1 on total c-Myc levels or c-Myc-Ser62 phosphorylation in this cellular context, nor was there an effect on phosphorylation of AKT-Thr308, AMPKα-Thr172, and p70S6K-Thr389 through HES1.

Notch1 Knockdown Has No Effect on PP2A Subunit Levels or PP2A in Vitro Catalytic Activity—ICN1 can be envisaged to activate transcription of genes encoding the A, B, and/or C PP2A subunits, providing a possible explanation for the effects of Notch1 on phosphorylation of diverse PP2A substrates. Thus, inhibition or knockdown of Notch1 could conceivably result in decreased levels of the individual PP2A subunits. By real-time PCR, relative transcript levels of the major catalytic α isomorph of the C subunit, PPP2CA, and of the B55α (PPP2R2A), B56β (PPP2R5B), and B56γ (PPP2R5C) regulatory subunits were not significantly different between the N1KD7 and NTC cells despite substantially reduced Notch1 activity in N1KD7 cells (Fig. 5A). Antibodies for B56β and B56γ were unavailable, so we could only assess protein levels for B55α, which were also unchanged (Fig. 5B). Likewise, the PP2A A and C subunits were unchanged between NTC and N1KD7 cells despite the substantial loss of Notch1 and increased phosphorylation of AKT-Thr308 (Fig. 5B). Analogous results were obtained in wild-type Jurkat cells treated with cpd-E (data not shown).

To measure PP2A catalytic activity, we used a commercial assay kit (Millipore) for which PP2A activity was reflected as liberation of free phosphate from a PP2A-specific phosphopeptide substrate using immunoprecipitated PP2A protein subunit C. Consistent with our Western blotting results, there was no difference in PP2A phosphatase activity between the NTC (607.5 ± 54.3 pmol of phosphate/min) and N1KD7
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As proof of concept, we considered the possibility that increased phosphorylation of AKT-Thr308, accompanying loss of Notch1, might be a reflection of decreased PP2A binding with AKT. PP2A was immunoprecipitated from both N1KD4 and N1KD7 cell homogenates with a monoclonal antibody directed against its catalytic C subunit, and its association with AKT was examined by Western blotting in triplicate experiments (Fig. 6). Signals were quantified by densitometry, and the results were recorded as ratios of AKT to PP2A (AKT/PP2A) in the immunoprecipitates. By this sensitive metric, total AKT was significantly decreased in the N1KD4 (0.409 (0.0103) p = 0.0046) cells and in the N1KD7 (0.530 (0.0524) p = 0.0009) cells as compared with NTC (arbitrarily set to a value of 1) cells. The decreased association of AKT with PP2A in the two clones was unbiased by the input and was specific to the PP2A IP because neither AKT nor PP2A was detected in mock IPs using normal mouse IgG (Fig. 6). Thus, AKT exhib-
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**FIGURE 6.** The association of AKT with PP2A is impaired accompanying the loss of Notch1. A and B, PP2A catalytic subunit (Sub C) was immunoprecipitated from untreated N1KD4 cell (A) or N1KD7 cell (B) homogenates and compared with the NTC cell homogenates in each experiment. The levels of the co-associated total AKT were analyzed by Western blotting. Immunoreactive proteins were quantified by measuring the intensities (background-corrected) of the bands by densitometry using the Odyssey (V3.0) software (LI-COR). The fractions of AKT associated with PP2A in the immunoprecipitates are expressed as ratios of total AKT to PP2A catalytic subunit protein (AKT/PP2A) for the N1KD4 (0.409 (± 0.103) (p = 0.0046) cells (chart to the right of panel A) and N1KD7 (0.530 (± 0.0524) (p = 0.0009)) cells (chart to the right of panel B), as compared with NTC cells (arbitrarily set to a value of 1). Lanes are designated as follows for the NTC (odd numbered lanes) and N1KD sublines (even numbered lanes): lanes 1 and 2, input (10 μg (4%)); lanes 3 and 4, mock IgG IP (250 μg); and lanes 5 and 6, PP2A catalytic subunit IP (250 μg). The insets to the left of lanes 1 and 2 are for different exposures of the input samples to more clearly reveal the relative differences in protein levels. Results are expressed as mean values ± standard errors from three independent experiments. p values equal to or less than 0.05 (95% confidence interval) were considered to be statistically significant (denoted by asterisks). The approximate molecular masses for immunoreactive proteins were: PP2A catalytic subunit (36–38 kDa), AKT (60 kDa), and normal mouse IgG (53 kDa).

**DISCUSSION**

Although effects of Notch1 on AKT signaling have been reported, both their direction and their magnitude have been variable in T-ALL (30, 46). The present study provides important new insights into the role of Notch1 in regulating AKT, aside from its well established capacity to transcriptionally regulate PTEN (30). Mutational loss of function (19) and post-translational inactivation of PTEN (32) are frequent events in T-ALL, promoting chronic AKT activation and GSI resistance (30).

In this study, we documented a previously unrecognized role for Notch1 in repressing AKT in PTEN-null, GSI-resistant T-ALL cells. Thus, treatment of Jurkat cells with GSI, cpd-E and DAPT, inhibited Notch1 and increased phosphorylation of AKT-Thr308 and to a lesser extent AKT-Ser473. This correlated with increased downstream signaling, as reflected in greater phosphorylation of GSK3α/β and FOXO1, which was blocked by a specific AKT inhibitor, perifosine (44). Increased AKT-Thr308 and GSK3α/β phosphorylation was also observed in CCRF-CEM and MOLT3 cells, both of which are GSI-resistant. Further, an identical phenotype was observed in Jurkat N1KD4 and N1KD7 sublines in which Notch1 was stably knocked down with lentiviral shRNA. There was no effect of Notch1 knockdown on cell proliferation or on cell cycle profile.

Our finding that in the absence of wild-type PTEN, Notch1 inhibits AKT signaling is inconsistent with published studies that established that Notch1 targets activate (HES1 (PTEN-dependent), IGF1R, and IL7R) or indirectly stabilize (miRNA-709) AKT (5, 14). Rather, our results strongly suggest the involvement of a unique mechanism for AKT inhibition by Notch1, which may not have otherwise been observed in cells expressing wild-type PTEN. The differential effects of the PI3K inhibitor wortmannin on AKT-Thr308 phosphorylation between N1KD7 and NTC cells, or for untreated and cpd-E-treated Jurkat cells, suggested a PI3K-independent mechanism that is best explained in terms of Notch1 regulating AKT-Thr308 dephosphorylation.

A role for PP2A was suggested by treating cells with the selective PP2A inhibitor, OA (38). Thus, treatment with OA preferentially increased AKT-Thr308 phosphorylation in NTC cells with only a modest effect on N1KD7 cells, most likely due to already reduced PP2A function resulting from the Notch1 knockdown. Analogous results were seen with wild-type Jurkat cells treated with cpd-E and OA (data not shown). Although
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PHLPP might also contribute, this is not easily reconciled with results of our OA experiments because OA does not affect PHLPP activity (47). Of course, PHLPP and/or mTOR2 might easily play a role in regulating AKT-Ser473 phosphorylation levels, which were increased in N1KD7 and cpd-E-treated Jurkat cells but are not affected by OA. Further work is needed to establish factors promoting the increased AKT-Ser473 phosphorylation in the context of Notch1 inhibition and in the absence of functional PTEN. Notably, phosphorylation of other established PP2A targets including AMPKα-Thr172 and p70S6K-Thr389 was also increased in N1KD7 cells and in Jurkat cells treated with cpd-E, further supporting the notion that decreased PP2A dephosphorylation was causal. Not all PP2A substrates (i.e. phospho-c-Myc-Ser62) were equally affected.

The regulation of c-Myc is extraordinarily complex, involving both transcriptional and posttranscriptional mechanisms that may affect c-Myc levels and stability independent of Notch1 (13, 56). ICN1 transcriptional regulation of c-Myc has been primarily studied in PTEN-positive T-ALL cells lines (13), but results in PTEN-null cells have been variable (17). In PTEN-positive T-ALL cells, GSI treatment results in decreased c-Myc protein (13, 17). Under these conditions, PTEN can negatively regulate c-Myc protein at the posttranslational level, via inhibition of AKT and activation of GSK3β (56). GSK3β, in turn, phosphorylates c-Myc at Thr58, resulting in its proteasomal degradation when additionally phosphorylated at Ser62 (17, 56). In PTEN-null cells, such as Jurkat, increased AKT activity inhibits GSK3β and could prevent c-Myc degradation, as was observed in the N1KD7 cells. Although cpd-E treatment resulted in decreased c-Myc, this may reflect differences between chronic and acute loss of Notch1 signaling, including differential effects on the balance between decreased c-Myc transcription and increased stability.

ICN1 typically functions as a transcriptional activator, although transcriptional repression can also occur downstream of ICN1, mediated by its effectors such as HES1 (9, 51). A transcriptional mechanism was confirmed for the PP2A response by transient transfections of Jurkat cells with a dominant-negative MAML1 construct that interferes with ICN1 transactivation of Notch1 targets (33). This increased phosphorylation of AKT-Thr308, AMPKα-Thr172, and p70S6K-Thr389, analogous to the effects of Notch1 knockdown. In addition, phosphorylations of AKT-Thr308, AMPKα-Thr172, and p70S6K-Thr389 were all dramatically decreased when N1KD7 cells were transfected with HES1. Although these results suggest that a Notch1 transcriptional program is responsible for the increased phosphorylation of these PP2A substrates, this is independent of changes in levels of transcripts or proteins for the major PP2A substrates, or PP2A catalytic activity, as measured with a phospho-peptide substrate.

Rather, our data imply that Notch1 may regulate PP2A substrate specificities, as suggested by the increased phosphorylation of AKT-Thr308, AMPKα-Thr172, and p70S6K-Thr389, but not c-Myc-Ser62. Three independent immunoprecipitation assays using both the N1KD4 and the N1KD7 cells demonstrated significantly decreased AKT associations with PP2A, thus explaining the increased AKT-Thr308 phosphorylation upon loss of Notch1 signaling. Although determinations of whether Notch1 similarly regulates PP2A associations with AMPKα and p70S6K will require further study, an analogous mechanism was strongly implied by findings of increased phosphorylation of AMPKα-Thr172 and p70S6K-Thr389 upon loss of Notch1.

Our finding that Notch1 knockdown increased phosphorylation of p70S6K-Thr389 in addition to AKT-Thr308 and AMPKα-Thr172 is inconsistent with the established role of AMPKα as a negative regulator of mTOR1 signaling and of p70S6K-Thr389 phosphorylation, although a role for AKT in activation of mTOR1 cannot be ruled out (19). Further, AKT and AMPK are normally antagonistic as activated phospho-AMPKα-Thr172 indirectly facilitates AKT dephosphorylation, whereas increased AKT signaling phosphorylates AMPK at Ser485/492, permitting dephosphorylation of Thr172 (57). These anomalies may reflect posttranslational modifications of PP2A regulatory (B55α, B56β, B56γ) or catalytic subunits that impact specificities for PP2A substrates and/or PP2A catalytic activity (55). Thus, AMPK phosphorylation of the B56γ PP2A subunit increases PP2A dephosphorylation of AKT (54). p70S6K-Thr389 is also regulated by B56γ (26).

By extension, mechanisms involving posttranslational regulation of PP2A can be envisaged to explain the apparent discrepancy involving PP2A levels and loss of PP2A activity toward phospho-AKT-Thr308 (and possibly phospho-AMPKα-Thr172 and phospho-p70S6K-Thr389), resulting from Notch1 knockdown or GSI inhibition. Indeed, Notch1 can be envisaged to directly or indirectly regulate posttranslational modifications of PP2A involving one or more of its subunit constituents. Notch1 was recently suggested to associate with the Src kinase, p56lc (58), which was reported to phosphorylate and inactivate PP2A (59). Although phosphorylation of the PP2A catalytic subunit controls its association with regulatory subunits, the deleterious effect of this modification on in vitro PP2A activity does not agree with results of our in vitro activity assays. An intriguing possibility involves regulation of PP2A methylation (reviewed in Ref. 55), which in turn regulates PP2A substrate specificities without compromising catalytic activity (60) and by affecting B subunit (B55α and B56 family) association with PP2A holoenzyme. Methylation by leucine carboxyl methyltransferase 1 (LCMT1) and demethylation by protein methyltransferase-1 (PME-1) regulate PP2A association with B55α and subsequent dephosphorylation of AKT and p70S6K (61). Alternatively, phosphorylation of the B subunits could impact substrate specificities by regulating their associations with PP2A and/or PP2A substrates (55). Both AMPK and extracellular signal-regulated kinase (ERK) have been reported to regulate B56γ phosphorylation (22, 54, 62). The dual specificity protein kinase Clk2 can phosphorylate B56β, which is important for the PP2A holoenzyme to assemble onto AKT (49). These possibilities are currently under investigation.

In summary, our results establish an intriguing and unprecedented role for Notch1 in regulating phosphorylation of AKT-Thr308, AMPKα-Thr172, and p70S6K-Thr389, all PP2A substrates, and apparently mediated at least in part by HES1. Mechanistically, we found that Notch1 knockdown decreased the level of AKT associated with PP2A, providing an explanation for its impaired dephosphorylation by PP2A. Although the detailed mechanisms responsible for increased phosphoryla-
tion of other PP2A targets will require further study, data presented herein strongly argue for a role for PP2A. Increased phosphorylations of all these PP2A substrates were unrelated to the absolute levels of PP2A catalytic, structural, or regulatory subunits, although an effect of Notch1 on PP2A posttranslational modifications is certainly possible. Clearly, better understanding of the responsible cellular and molecular regulatory mechanisms that impact Notch1 and AKT signaling may lead to rational new therapies for T-ALL that target these critical pathways.

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