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Loss of Oncogenic Notch1 with Resistance to a PI3K Inhibitor in T Cell Leukaemia

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Abstract

Mutations that deregulate Notch1 and Ras/PI3 kinase/Akt signalling are prevalent in T lineage acute lymphoblastic leukaemia (T-ALL), and often coexist. The PI3 kinase inhibitor GDC-0941 was active against primary T-ALLs from wild-type and $Kras^{G12D}$ mice and addition of the MEK inhibitor PD0325901 increased efficacy. Mice invariably relapsed after treatment with drug resistant clones, most of which unexpectedly had reduced levels of activated Notch1 protein, down-regulated many Notch1 target genes, and exhibited cross-resistance to γ secretase inhibitors. Multiple resistant primary T-ALLs that emerged *in vivo* did not contain somatic *Notch1* mutations present in the parental leukaemia. Importantly, resistant clones up-regulated PI3K signalling. Consistent with these data, inhibiting Notch1 activated the PI3K pathway, providing a likely mechanism for selection against oncogenic Notch1 signalling. These studies validate PI3K as a

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therapeutic target in T-ALL and raise the unexpected possibility that dual inhibition of PI3K and Notch1 signalling could facilitate drug resistance in T-ALL.

Introduction

Targeted anti-cancer therapies exploit genetic and biochemical alterations unique to malignant cells, and administering combinations of targeted drugs based on the constellation of specific mutations present in an individual tumour is a rational strategy for treating advanced cancers. In T-ALL, mutations that deregulate Notch1 and Ras/PI3K signalling occur in 60% and 55% of patients, respectively [1-3]. Recent data indicate that PI3K pathway activation is associated with aggressive biological features, drug resistance and poor prognosis in T-ALL[4-9]. GDC-0941 is a potent PI3K inhibitor (PI3Ki) that is rapidly advancing in clinical development[10]. We observed down-regulation of activated Notch signalling and reduced Myc expression in T-ALL cell lines and primary leukaemias that became resistant to GDC-0941 *in vitro* and *in vivo*. These data implicate PI3K as a dominant oncogenic driver pathway in T-ALL, and indicate that combining targeted anti-cancer drugs may have unanticipated effects on clonal dynamics and inadvertently promote the outgrowth of resistant cells.

PI3Ki Resistance Involves Notch1 Inactivation

T-ALLs were generated by performing retroviral insertional mutagenesis (RIM) in WT (*Kras*^{WT}) and *Mx1-Cre, Kras*$^{LSL-G12D}$ (*Kras*^{G12D}) mice[11] and by expressing mutant *Kras* in a transduction/transplantation system[12]. These primary T-ALLs are characterized by diverse retroviral integrations, heterogeneous biochemical activation of the Raf/MEK/ERK and PI3K/Akt effector pathways, and secondary acquisition of somatic *Notch1* mutations (**Extended Data Fig. 1a**). Cell lines generated from murine T-ALLs are uniformly sensitive to PI3K inhibition[11,12]. To uncover potential mechanisms of acquired resistance, we exposed T-ALL cell line E2[12] to increasing concentrations of GDC-0941[10]. In comparison to the parental E2 cell line, all three resistant lines (E2-R3, E2-R5, and E2-R6) proliferated in 10-fold higher concentration of GDC-041 as assessed by cell numbers (**Fig. 1a**) and 5-bromo-2'-deoxyuridine (BrdU) labelling (**Fig. 1b** and **Extended Data Fig. 2a**). Resistant E2 cells also required higher drug concentrations to efficiently induce cleaved caspase 3 and inhibit higher levels of phosphorylated Akt (pAkt) (**Figs. 1c, 1d**). Unexpectedly, resistant E2 clones down-regulated the expression of activated Notch intracellular domain (NICD) proteins and were insensitive to Compound E, a potent γ secretase inhibitor (GSI) that blocks an essential enzymatic cleavage step for generating NICD[3] (**Figs. 1e, 1f**).

Efficacy of PI3K and MEK Inhibitors *In Vivo*

Leukaemias generated by RIM recapitulate the genetic heterogeneity seen in advanced human cancers, and transplantation into recipient mice provides a flexible system for investigating therapeutic responses and elucidating mechanisms of drug resistance (**Extended Data Fig. 1b)**. Advantages of this approach include: (1) primary cancers are treated in immunocompetent mice; (2) retroviral integration patterns can be used to track the emergence of drug resistant clones; and, (3) relapsed leukaemia cells can be re-transplanted

to verify intrinsic resistance and test alternative therapies[13]. We transplanted 6 $Kras^{WT}$ and 15 $Kras^{G12D}$ T-ALLs (**Extended Data Table 1**) into 168 recipients (8 per leukaemia) and randomly assigned these mice to receive GDC-0941 or control vehicle (n = 5 and 3, respectively). Mice were treated for 8 weeks or until they required euthanasia due to progressive leukaemia. The maximally tolerated dose (MTD) of GDC-0941 is 125 mg/kg/day in sub-lethally irradiated mice and results in drug exposures sufficient to effectively inhibit PI3K for >8 hours (**Extended Data Fig. 3**). Overall, treatment at this dose significantly extended the survival of recipients transplanted with $Kras^{WT}$, but not $Kras^{G12D}$, leukaemias (**Figs. 2a, 2b**). An analysis of individual primary leukaemias showed that 3 of 6 $Kras^{WT}$ and 4 of 15 $Kras^{G12D}$ T-ALLs responded to GDC-0941 (**Extended Data Table 1**). Importantly, these heterogeneous and transient *in vivo* responses contrast with the uniform *in vitro* sensitivity of T-ALL cell lines to PI3K inhibition.[11,12]

The Raf/MEK/ERK pathway is a major effector of oncogenic Ras signalling, and combining MEK and PI3K inhibitors is efficacious in murine *Kras*-driven tumours[14]. We previously administered PD325901 (PD901), a potent and selective MEK inhibitor[15], to mice transplanted with $Kras^{WT}$ or $Kras^{G12D}$ T-ALLs[11]. PD901 marginally increased the survival of recipients of $Kras^{G12D}$ leukaemias (median 3 days), but was not efficacious in $Kras^{WT}$ T-ALLs[11]. Treating mice with the same dose of PD901 (5 mg/kg/day) and GDC-0941 at the MTD of 125 mg/kg/day caused weight loss and severe anaemia (data not shown). To reduce toxicity, we devised a protocol whereby mice received daily GDC-0941 (100 mg/kg/day) as a “backbone” in combination with intermittent PD901 (5 mg/kg given 4 days per week; see **Extended Data Fig. 1c)**. This new regimen was evaluated in a second randomized trial that included 136 recipients of 12 $Kras^{G12D}$ and 5 $Kras^{WT}$ T-ALLs investigated in the previous single agent trial of GDC-0941 (**Extended Data Table 1**). Treatment with the GDC-0941/PD901 combination dramatically extended the survival of mice transplanted with either $Kras^{WT}$ or $Kras^{G12D}$ T-ALLs compared to the control vehicle (**Figs. 2a, 2b**). Examining the individual $Kras^{G12D}$ leukaemias showed that the GDC-0941/PD901 combination markedly extended the survival of recipients transplanted with T-ALLs that were initially sensitive to GDC-0941, and was also efficacious in 7 leukaemias that were unresponsive to GDC-0941 alone (**Extended Data Table 1**).

Drug Resistant Clones Down-Regulate NICD

Despite continuous treatment, recipient mice invariably died of refractory T-ALL, which showed similar morphologic and immunophenotypic features as the respective parental leukaemia (**Extended Data Fig. 4**). We transplanted leukaemia cells from mice that succumbed after initially responding to either GDC-0941 (n = 5) or to the GDC-0941/PD901 combination (n = 5) into secondary recipients, retreated them, and found that none responded *in vivo* (**Fig. 2c**). Based on these functional data, we hereafter refer to leukaemias that emerged after an initial response to targeted agents as “resistant”. We analysed retroviral integrations to ask if resistant T-ALLs exhibit clonal evolution. Southern blotting with a virus-specific probe revealed shared restriction fragments as well as one or more novel bands in many resistant leukaemias (**Fig. 2d**), and shotgun cloning of viral integration sites demonstrated insertions that were unique to or highly enriched in different resistant subclones (**Extended Data Table 2**). Clonal evolution was observed in 10 of 14 responsive

T-ALLs, but not in the 7 primary T-ALLs that displayed *de novo* drug resistance (**Extended Data Table 1**).

Reduced NICD expression in GDC-0941-resistant cell lines **(Fig. 1e)** led us to examine Notch1 signalling in resistant T-ALLs that emerge *in vivo.* Indeed, Western blot analysis revealed greatly reduced NICD expression in 19 of 31 (61%) independent resistant clones derived from 8 parental T-ALLs (**Fig. 3a and Extended Data Fig. 5a**). Resistant T-ALLs also showed markedly reduced levels of Myc, a downstream effector of activated Notch signalling strongly implicated in leukaemogenesis (**Fig. 3a**)[1]. Expression profiling of 17 "NICD/Myc off" resistant T-ALLs demonstrated down-regulation of multiple Notch1 transcriptional target genes including *Notch1, Notch3, Myc, Ptcra, Dtx1,* and *Il2ra* compared to the corresponding parental leukaemia (**Fig. 3b**). Interestingly, *Nrarp* and *Hes1* transcript levels were stable or higher in resistant T-ALLs, a finding independently verified by quantitative real-time PCR (**Fig. 3b** and data not shown). This observation suggests that *Nrarp* and *Hes1* expression is uncoupled from Notch1 pathway activation in resistant T-ALLs.

To determine if resistant leukaemias are less dependent on Notch1 signalling, we exposed primary T-ALL cells to Compound E *ex vivo.* Multiple resistant clones isolated from two parental T-ALLs showed reduced sensitivity to GSI treatment than the corresponding parental leukaemia (**Figs. 3c** and **Extended Data Fig. 5b**). We also cultured parental and resistant leukaemia cells in a range of GDC-0941 concentrations with or without 0.1 μM of Compound E. Whereas Compound E cooperated with GDC-0941 to reduce the growth of parental T-ALLs, GSI treatment had no additive effects in the resistant clones (**Fig. 3d** and **Extended Data Fig. 5c)**.

We sequenced *Notch1* and found that mutations identified in the parental T-ALLs were unexpectedly absent or detected at greatly reduced levels in 6 independent resistant leukaemias derived from 4 parental T-ALLs (**Fig. 3e**). In addition, retroviral integrations within the Notch pathway genes *Notch1, Aph1,* and *Dtx1* were highly enriched in three resistant leukaemias with reduced NICD/Myc expression, strongly implicating insertional mutagenesis as an alternative mechanism of reduced Notch1 signalling (**Extended Data Fig. 5d and Extended Data Table 2**). Importantly, resistant leukaemias with decreased or absent NICD and Myc expression were recovered from recipients treated with either GDC-0941 or with the GDC-0941/PD901 combination, suggesting that P13K inhibition imposes selective pressure to down-regulated oncogenic Notch1 signalling.

Activated Notch1 Supresses PI3K Signalling

To address why *Notch1* mutations that confer a clonal growth advantage during leukaemogenesis might unexpectedly "switch" to become deleterious when PI3K is inhibited therapeutically, we examined PI3K signalling in resistant T-ALLs with loss of NICD expression. Each parental and resistant T-ALL displayed a consistent pattern of protein phosphorylation in independent recipient mice (**Fig. 4a**). Resistant leukaemias generally had reduced PTEN expression and biochemical evidence of PI3K pathway activation characterized by elevated pAkt and pS6 levels relative to the respective parental

T-ALL (**Fig. 4a**). Some resistant leukaemias also increased pERK (**Fig. 4a**). Whereas GDC-0941 treatment markedly reduced BrdU incorporation by parental T-ALLs *in vivo,* resistant leukaemias continued to proliferate (**Fig. 4b** and **Extended Data Fig. 2b**). Parental T-ALL cells isolated from recipient mice expressed high levels of cleaved caspase 3 after overnight culture, which were further induced by exposure to GDC-0941 (**Fig. 4c**). Basal cleaved caspase 3 levels were remarkably lower in resistant T-ALLs, but were induced by GDC-0941 exposure (**Fig. 4c**). Importantly, induction of cleaved caspase 3 in resistant T-ALLs correlated with the 5-50 fold higher concentrations of GDC-0941 required to suppress pAkt (**Fig 4d and Extended Data Fig. 6**).

In T-ALL and during thymic development Notch1 up-regulates *Hes1*, which enhances PI3K signalling by inhibiting *PTEN* expression[6,16]. These data support the existence of selective pressure for resistant T-ALLs to retain *Hes1* expression, even as they down-regulated other Notch1 target genes (**Fig. 3b**). We reasoned that outgrowth of leukemic clones lacking NICD might reflect additional inhibitory crosstalk between these two oncogenic pathways. To address this question, we first asked if reducing Notch1 activity modulates PI3K signalling in cell lines without PTEN expression. Indeed, exposing PTEN deficient cell lines to Compound E induced Akt phosphorylation (**Fig. 4e**). This response was not observed in BW, a T-ALL cell line lacking NICD expression (**Fig. 4e**), and was variable in T-ALL cell lines with intact PTEN expression (**Extended Data Fig. 7a, b**). The reciprocal experiment of pharmacologically inhibiting PI3K signalling did not alter NICD expression (**Extended Data Fig. 7c**).

We next modulated Notch pathway activation in Jurkat T-ALL cells to assess the effects on GDC-0941 responses. Enforced expression of NICD enhanced sensitivity to GDC-0941 (**Fig. 4f**), which was also true in several other T-ALL cell lines (**Extended Data Fig. 7d**). By contrast, expressing a dominant negative Mastermind protein (dnMAML) that blocks Notch1 transcriptional activity conferred resistance to GDC-0941 and increased pAkt levels (**Fig. 4f, 4g)**. Consistent with these data, exposing Jurkat cells to 0.1 μM of Compound E, which inhibits NICD but does not alter proliferation, antagonized GDC-0941-mediated growth inhibition (**Extended Data Fig. 7e, 7f**). Importantly, exogenous NICD expression abrogated the ability of Compound E to induce resistance to GDC-0941 (**Extended Data Fig 7g**).

Discussion

To our knowledge, this is the first example in which *in vivo* treatment with a targeted anti-cancer agent selected for resistant clones lacking activation of a major oncogenic pathway involved in tumourigenesis (**Fig 4h**). Parental leukaemias expressing NICD activate Myc and other Notch1 target genes (**Fig 4h, left panel**). Many of these aggressive cancers respond to GDC-0941 – either as a single agent or in combination with a MEK inhibitor. However, all ultimately develop drug resistance, which is frequently characterized by loss of NICD expression and enhanced activation of PI3K/Akt/S6 signalling pathway (**Fig 4h, right panel**). Biochemical and transcriptional down-regulation of Notch1 signalling occurred in over 60% of resistant T-ALLs. The late acquisition of Notch1 mutations in murine T-ALLs[11,12] is consistent with human studies showing that *NOTCH1* mutations are frequently

cooperating events that may be absent at relapse[17]. Similarly, this RIM model accurately recapitulates the branched clonal architecture observed in relapsed human leukaemia[18] as resistant T-ALLs isolated after *in vivo* drug treatment invariably shared multiple retroviral insertions with the corresponding parental T-ALL (**Fig. 2d**). In some instances, isolating the same resistant T-ALL from multiple independent recipient mice provided compelling evidence for treatment-induced selection of a pre-existing clone (**Fig. 2d**). However, MOL4070LTR is a replication competent retrovirus and new integrations might also contribute to resistance.

We show that resistant T-ALLs restore activated PI3K/Akt signalling, but remain sensitive to high concentrations of GDC-0941. This paradigm of “on pathway” but “off target” resistance has been observed in advanced human cancers that recurred after treatment with tyrosine kinase inhibitors. For example, melanomas treated with B-Raf kinase inhibitors deploy multiple genetic mechanisms to reactivate Raf/MEK/ERK signalling (reviewed in[19]). It is therefore theoretically possible that more potent or selective PI3K inhibitors could overcome acquired resistance in T-ALL, However, we administered GDC-0941 at the MTD and deeper target inhibition might not be feasible due to toxicity to normal tissues.

Resistant T-ALL cells down-regulated Myc expression. As Myc directly binds and positively regulates the *PTEN* promoter in T-ALL[6], loss of Myc expression might facilitate the outgrowth of GDC-0941-resistant cells through this mechanism. However, *PTEN* negative T-ALL cell lines increased pAkt levels in response to Compound E, which infers the existence of PTEN-independent inhibitory crosstalk between the Notch1 and PI3K/Akt pathways. This idea is consistent with a recent report showing that Notch1 inhibits Akt activation by enhancing association with the PP2A phosphatase in Jurkat cells[20]. Furthermore, enforced expression of Myc did not recapitulate the enhanced sensitivity to GDC-0941 induced by NICD (**Fig. 4f, Supplemental Fig 7d**, and data not shown). While it is unclear how resistant T-ALL cells compensate for loss of Myc expression, *PTEN* loss or Akt activation overcomes the adverse effects of Myc withdrawal in a zebrafish model of T-ALL[21]. The unexpected observation that elevated NICD and Myc expression is dispensable for T-ALL growth *in vivo* might partially underlie the disappointing clinical efficacy of GSIs observed to date[22].

GDC-0941 prolongs the survival of recipient mice transplanted with primary T-ALL cells and drives clonal evolution *in vivo,* thereby validating PI3K as a therapeutic target in this aggressive cancer. Together with a recent study implicating PI3K signalling in resistance to glucocorticoids[5], our data strongly support evaluating PI3K inhibition in T-ALL in combination with other anti-leukaemia agents. *RAS* mutations are commonly detected in early T-cell precursor ALL, an aggressive paediatric leukaemia with a high rate of treatment failure[23], and a recent study implicated *NRAS* mutations in T-ALL relapse[24]. We identified oncogenic *Kras* mutations as a biomarker of resistance to GDC-0941 mono-therapy, and provide evidence that combining PI3K and MEK inhibitors might be beneficial in these challenging patients. Simultaneously targeting the Notch1 and PI3K/Akt pathways is a rational therapeutic approach in T-ALL[6,25]. Importantly, however, our data suggest that combination regimens might inadvertently undermine the efficacy of PI3K inhibitors by facilitating the survival and outgrowth of drug resistant clones. Given this, sequential

treatment with a Notch1 pathway inhibitor to suppress *NOTCH1* mutant clones found at diagnosis followed by PI3K/Akt inhibition is an appealing approach. Finally, whereas the *NOTCH1* mutations found in T-ALL constitutively increase Notch1 signalling, inactivating mutations are implicated as "drivers" in other human cancers[26]. Our studies therefore raise the provocative possibility that these loss-of-function *NOTCH1* mutations contribute to tumourigenesis by aberrantly activating PI3K signalling.

METHOD SUMMARY

GDC-0941 was provided by Genentech, Inc (South San Francisco, CA) and synthesized as previously described[14]. Compound E was purchased from Calbiochem. The PD0325901 used in these studies was synthesized by Shanghai Chempartner. All drugs were diluted in DMSO for *in vitro* studies. To generate resistant cell lines, the E2 line was plated in 0.5 μM GDC-0941 in 6 individual wells. As the cells became confluent, they were passaged and the GDC-0941 dose was increased to generate resistant clones E2-R3, E2-R5, and E2-R6, all of which grow in lymphocyte growth medium (LGM) containing 60 μM GDC-0941.The *Notch1* gene was amplified using primers 5'-ATAGCATGATGGGGCCACTA-3' and 5'-CTCTGGAATGTGGGTGATCTGG-3' as previously described[11]. Southern Blotting and cloning of retroviral insertions were performed as described elsewhere[11]. PCR products were sequenced by Quintara, and retroviral sequence tags were aligned against the UCSC mm9 mouse reference genome assembly. All animal experiments conformed to national regulatory standards and were approved by the UCSF Committee on Animal Research. For T-ALL transplantation, 2×10^6 cells were injected retro-orbitally into male 8-12 week old sublethally irradiated congenic recipient mice. For pharmacokinetic and pharmacodynamic studies, plasma was isolated from blood and the concentrations of GDC-00941 in each plasma sample were determined by a non-validated LC/MS/MS assay as previously described[14]. Total Akt and p-Akt S473 were measured by the Multi-Spot Detection System (Meso Scale Discovery). For preclinical testing, recipient mice were randomly assigned to receive control vehicle (0.5% hydroxypropyl methylcellulose and 0.2% Tween 80), GDC-0941 as a single agent, or GDC-0941 combined with PD0325901 4 days per week. We performed gene expression profiling on 28 independent parental and resistant mouse T-ALL tumours (**Extended Data Table 3**) using Affymetrix GeneChip HT MG-430 PM Affymetrix microarrays (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions.

Methods

Plasmids

pRIEN (a generous gift of Drs. Johannes Zuber and Scott Lowe) was used to introduce the ecotropic receptor into human cell lines to enable efficient retroviral infection and is Neo-resistant version of pRIEP[27]. MIG (MSCV-IRES-GFP) is the murine stem cell virus (MSCV) vector containing a green fluorescent protein (GFP) cassette driven by an internal ribosome entry site (IRES)[28]. MIG-NICD[29] and MSCV-dnMAML-eGFP[30,31] have been described previously.

Cell Culture

T-ALL cell line E2[12] was maintained in lymphocyte growth medium (LGM; DME-H21, 20% FBS, 10mM Hepes, penicillin/streptomycin, L-glutamine, non-essential amino acids, sodium pyruvate, 50μM β-mercaptoethanol 10ng/mL IL-2, and 10ng/mL IL-7). Cell lines BW (BW5147.3; #TIB-47), S1A (S1A.TB.4.8.2; #TIB-27), WEHI (WEHI7.1; #TIB-53) and R1.1 (#TIB-42) were purchased from the ATCC. Jurkat cells were stably infected with pRIEN to allow retroviral infection. RIEN-expressing Jurkat cells were infected with MIG, MIG-NICD or MSCV-dnMAML-eGFP and sorted for GFP. All cell lines were grown and maintained as recommended by the ATCC and tested every 6 months for mycoplasma.

Cell Line Drug Escalation

The E2 cell line was plated in 0.5 μM GDC-0941 in 6 individual wells. As cells became confluent, they were passaged and GDC-0941 dose was increased to generate resistant clones E2-R3, E2-R5, and E2-R6, all of which grow in LGM containing 60 μM GDC-0941.

Proliferation Assays

For cell line proliferation assays, 3-5 × 10^5 cells per well were grown in LCM in 24-well/plate format. Drugs suspended in DMSO or an equal volume of DMSO only (vehicle control) were added to each well. After a minimum of four cell doublings in vehicle wells, the cells were harvested and counted on a Vi-Cell XR (Beckman-Coulter). For proliferation assays of T-ALL cell lines infected with MSCV-GFP viruses (REF), the cells were collected 72 hours after infection. GFP-positive and GFP-negative cells were co-cultured and proliferation assays were performed to compare the effects of drug treatment on each population. Proliferation was assessed as described above except live cells were counted by flow cytometry using 7-ADD exclusion and CountBright absolute counting beads (Invitrogen). For primary T-ALL proliferation assays, 2.5-5 × 10^5 cells per well were plated in triplicate in a 96 well plate. After 48 hours, cells number was assayed using CellTiter 96 MTS reagent (Promega). For Jurkat combination assays, 1 × 10^4 cells were plated in triplicate and MTS activity was assayed at 72 hours.

Western Blot Analysis

Cells were lysed in RIPA buffer containing Complete Protease Inhibitor Cocktail (Roche). anti-GFP antibody was from Santa Cruz Biotechnology (8334). All other antibodies were from Cell Signalling Technologies: pAkt S473 (4060), pAkt T308 (2965), Akt (9272), pErk (4370), pS6 (2211), Pten (9552), NICD (4147), cMyc (5605), and Actin (4967).

Genetic Analysis of T-ALL Cells

Genomic DNA was digested with Hind III, followed by electrophoresis, and capillary transfer to Hybond-N filters (Amersham). Filters were hybridized with a MOL4070 LTR probe that was labelled with radioactive α-dCTP using Rediprime II (Amersham). For *Notch1* mutation analysis, genomic DNA was amplified using primers 5'-ATAGCATGATGGGGCCACTA-3' and 5'-CTCTGGAATGTGGGTGATCTGG-3', which span exons X-Y of *Notch1* as previously described[11]. Retroviral integrations were cloned using a "shotgun" strategy exactly as described elsewhere [11]. The PCR products were

sequenced by Quintara, and sequence tags were aligned against the mouse reference genome assembly. First, we demanded sequence tags to have valid LTR sequence of MOL4070 virus using cross_match (http://www.phrap.org). Then, we trimmed the virus sequence from the sequence tags, and aligned them against the mouse reference genome (UCSC mm9 assembly) using BLAT[32]. We used sequence tags aligned to the reference genome with >90% identify and >90% coverage. The tags aligned to multiple genomic locus with nearly identical alignment scores were discarded as tags with non-unique alignment. Finally, we obtained independent virus insertion loci by consolidating the tags supporting the same integration sites. We used the number of supporting tags for each independent locus as the indicator of the virus insertion sites in the tumour cell population.

Mice

All animal experiments conformed to national regulatory standards and were approved by the UCSF Committee on Animal Research. For adoptive transfer of acute leukaemias, 2×10^6 cells were injected retro-orbitally into 8-12 week old wild-type C57Bl6/129Sv F1 recipient mice that had been irradiated with 450 cGy.

Pharmacokinetics and Pharmacodynamics

For pharmacokinetic measurements, FVB mice were treated with a single dose of GDC-0941 and blood collected at 0.5, 1, 2, 4, 8, 12 and 24 hours post dose by cardiac puncture. Plasma was isolated from blood and the concentrations of GDC-00941 in each plasma sample were determined by a non-validated LC/MS/MS assay in the Drug Metabolism and Pharmacokinetics (DMPK) Department at Genentech as previously described[14]. The assay lower limit of quantitation (LLOQ) was 0.005 < M. For pharmacodynamic measurement, bone marrow from a single mouse was harvested in DME media containing 1% BSA and treated with varying concentrations of GDC-0941 for 15 minutes prior to stimulation with 10 ng/ml GM-CSF for 10 minutes. Cell lysates were prepared by homogenization in cell extraction buffer (Invitrogen; Camarillo, CA), containing 10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate supplemented with phosphatase and protease inhibitors (Sigma, St. Louis, MO). Levels of total Akt, Akt phosphorylated at serine 473 (phospho-Akt) were measured by The Meso Scale Discovery Multi-Spot Biomarker Detection System (Meso Scale Discovery; Gaithersburg, MD) Phosphorylated protein were normalized to total protein levels in GDC-0941–treated samples and compared to vehicle controls.

Preclinical Trials

In the single agent trial, recipient mice were randomly assigned to receive GDC-0941 at a dose of 125 mg/kg/day by oral gavage or a control vehicle (0.5% hydroxypropyl methylcellulose and 0.2% Tween 80). For combination treatment, mice received 100 mg/kg/day of GDC-0941 daily and PD0325901 was administered by oral gavage at a dose of 5 mg/kg/day 4 days per week. Blinding was not feasible for these trials. Mice were observed daily during 8 weeks of treatment and weighed weekly to adjust the dose. Based on criteria established before initiating treatment, mice that died from causes other than leukaemia (diagnosed based in haematologic findings that include elevated WBC count,

splenomegaly, and lymphoblasts in the bone marrow) were censored. The time to death in recipient mice transplanted with the same primary leukaemias typically varies by <10%. This observation and previous work[13] established a sample size of 5 drug-treated and 3 vehicle-treated mice per leukaemia as having adequate statistical power to detect differences ≥ 50% in survival. To reflect the genetic heterogeneity present in the RIM system and in human leukaemia, we treated >20 individual $Kras^{WT}$ and $Kras^{G12D}$ leukaemias in over 250 individual mice that were randomly assigned to receive either control vehicle or drug treatment.

Expression Profiling and Informatics

28 mouse T-ALL tumours (**Extended Data Table 3**) were stained for CD4 and/or CD8 positive cells as described above and sorted on a FACSAriaIII (BD Biosciences) We performed gene expression profiling using Affymetrix GeneChip HT MG-430 PM Affymetrix microarrays (Affymetrix) according to the manufacturer's instructions. Signals from all samples were normalized using the Robust Multichip Average (RMA) algorithm implemented in Bioconductor R package[33]. Differential expression analysis was performed pairwise between all "Parental" and "Resistant" samples within each leukaemia (2M, 20M, etc). When 2 parental samples were available, resistant clones were compared independently to each, resulting in a total of 25 comparisons representing 17 unique resistant leukaemias. Fold change was calculated as the ratio of the gene expression of the two samples and a heatmap of expression fold change on Notch1 target genes was generated in R for visualization. Gene expression data have been deposited in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, accession number GSE48260).

Immunophenotyping

Staining was carried out in FACS Staining Buffer (PBS with 2% heat inactivated FCS). Primary leukaemia cells were stained with APC or PE anti-CD4 (clone GK1.5) and PE or PECy7 anti-CD8 (clone 53-6.7) antibodies (BD Biosciences) for 20 min, washed twice and analysed by flow cytometry using a FACSAriaIII or LSRII with FACSDiva software (BD Biosciences). Data were analysed using FlowJo software (TreeStar).

Cell cycle analysis

For *in vivo* labelling, mice were dosed with drug and 4 hours later 1 mg/10 g BrdU was administered by IP injection and allowed to incorporate for 2 hours. Cells were harvested and stained with FITC anti-CD4 and anti-CD8 antibodies prior to BrdU staining. Cell lines were plated in in DMEM containing 10% FBS and drug for 16 hrs then 10uM BrdU was added to the culture media and allowed to incorporate for 90 min. Staining with a V450 conjugated anti-BrdU antibody (clone 3D4) and 7-AAD was performed using a BrdU Flow Kit (BD Biosciences).

Apoptosis assays

Primary T-ALL cells were harvested from mice and plated in LGM containing drug for 8 hrs. Cell lines were plated in in DMEM containing 10% FBS and drug for 18 hrs. Cellular

staining with a V450 conjugated anti-cleaved Caspase3 antibody (C92-605) was performed using a Cleaved Caspase Flow Kit (BD Biosciences).

Statistics

Two-sided t test was used to evaluate differences between conditions and a normal distribution of data points around the mean was assumed based on the use of isogenic cell lines and genetically identical leukaemias in all experiments. Error bars representing S.E.M. indicate variation for each group and variance was found to be similar between compared groups. Log-rank analysis was used to evaluate differences in Kaplan-Meier Survival curves.

Extended Data

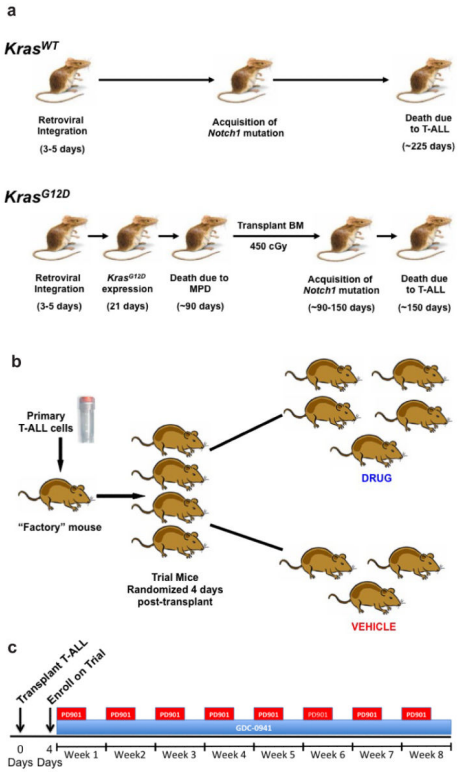

Figure ED1. Generation of primary T-ALLs and preclinical trial design

a, Neonatal *Kras*^{WT} and *Mx1-Cre, LSL-Kras*^{G12D} mice that were injected with the MOL4070LTR retrovirus at birth were observed until death as described in Ref 5. Half of *Kras*^{WT} T-ALLs harbour *Notch1* mutations. *Kras*^{G12D} expression was induced at age 21 days in *Mx1-Cre, LSL-Kras*^{G12D} mice, and they died from an aggressive myeloproliferative neoplasm around 90 days of age. Bone marrow harvested at the time of death induced T-ALL when transferred into sublethally irradiated recipient mice. All of these *Kras*^{G12D} T-ALLs harbour *Notch1* mutations that are acquired post-transplant. Modified from Ref 5. **b,** Primary leukaemias are first expanded *in vivo* (factory mouse), and are then transplanted into multiple recipient mice of identical strain background. These mice are then randomly assigned to treatment with vehicle (control arm) or experimental drugs (GDC-0941 or

GDC-0941/PD901). **c,** Overview of GDC-0941/PD901 treatment regimen. Mice were transplanted at day zero (D0) and started treatment on day 4 (D4). GDC-0941 was administered daily at a dose of 100 mg/kg (blue bar), and PD901 (5 mg/kg/day) was cycled 4 days on and 3 days off each week (red boxes). Treatment was continued for 8 weeks or until death.

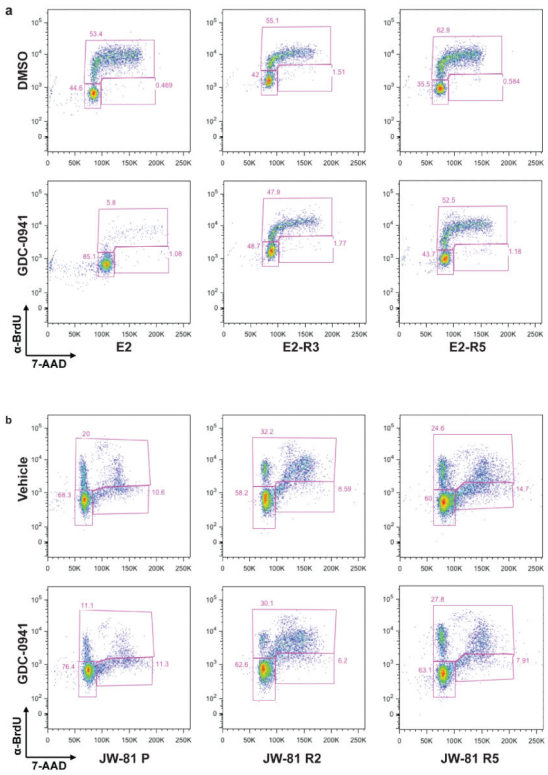

Figure ED2. Proliferation of parental and resistant E2 T-ALL cells and primary leukaemias

a, E2 cells were plated in DMSO or 1 μM GDC-0941 for 16 hr, labelled with 10 μM BrdU for 90 min, then analysed for BrdU incorporation and DNA content (7-AAD) by flow cytometry. The parental E2 line demonstrates a ~90% reduction (from 53.4 to 5.8%) in BrdU positive cells at this concentration of GDC-0941, while resistant lines (E2-R3 and E2-R5) continue to proliferate robustly. **b,** Recipient mice engrafted with T-ALL JW-81 parental and resistant clones were treated with GDC-0941 and injected with BrdU. *in vivo* treatment of these primary T-ALLs reduced the percentage of proliferating cells in the parental leukaemia by ~50% (from 20% to 11.1%), but had minimal effects on the two drug-resistant clones.

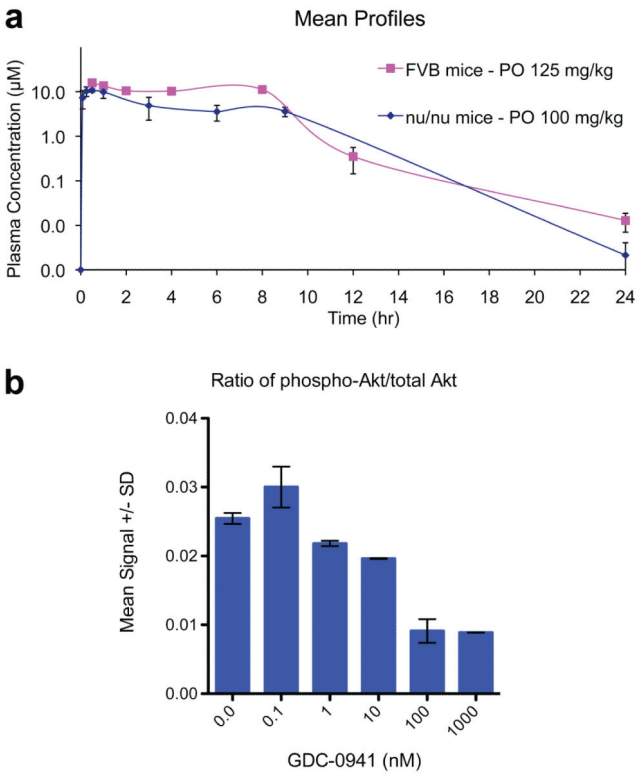

Figure ED3. Pharmacokinetics and pharmacodynamics of GDC-0941

a, Plasma concentration of GDC-0941 after a single dose in sv129/Blk6 F1 (FVB) mice and nu/nu mice (control). Each time point represents 3 mice and error bars show the S.E.M. **b,** Levels of Akt phosphorylation (pAkt) on serine 473 normalized to total Akt in murine bone marrow cells exposed to GDC-0941 *ex vivo* for 15 min before stimulation with 10 ng/ml GM-CSF. The IC^{90} for GDC-0941 is ~100 nM. Error bars show S.E.M. of 3 technical replicates.

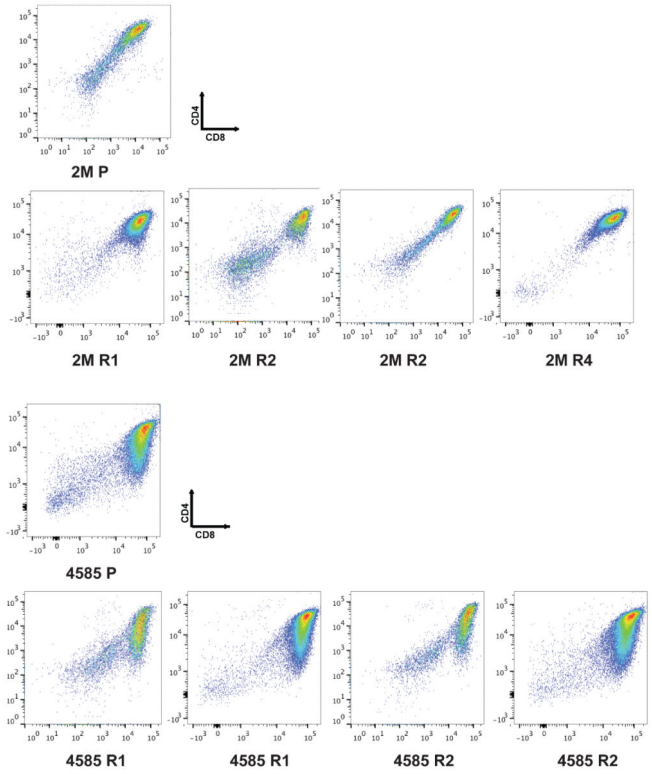

Figure ED4. Resistant T-ALLs retain the same immunophenotypes as the corresponding parental leukaemias

Flow cytometric analysis of leukaemia cells demonstrates identical patterns of CD4 and CD8 in parental and resistant leukaemias (n=4). Duplicates represent different transplant recipients.

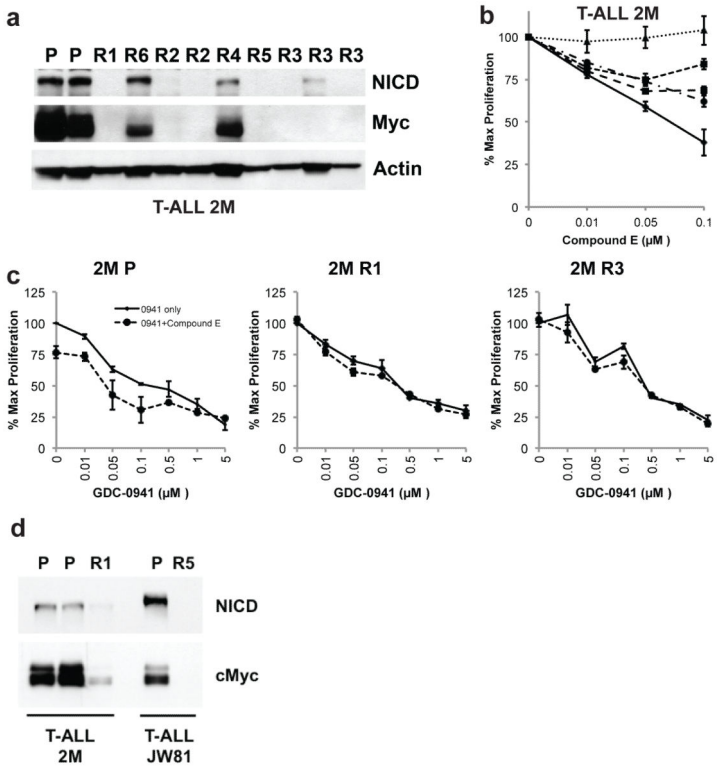

Figure ED5. GDC-0941 resistant leukaemias that emerge *in vivo* are cross-resistant to Compound E

a, Western blot analysis of 6 resistant clones (R1-R6) isolated at death from recipients that were transplanted with T-ALL 2M and treated. Note resistant T-ALLs R1-R3 & R5 have lost or markedly down-regulated NICD and Myc expression. Each lane contains bone marrow lysate isolated from an independent recipient of the indicated leukaemia (n = 2 for parental 2M, n = 2 for 2M R2, n = 3 for 2M R3). **b,c,** Parental T-ALL 2M and resistant leukaemias were exposed to Compound E (panel **b**) or GDC-0941 −/+ Compound E (panel **c**) *ex vivo* for 48 hours and proliferation was measured using an MTS assay. Each graph depicts % maximum MTS units and error bars are S.E.M. of 3 technical replicates **b,** Resistant leukaemias R1-R4 (dashed lines) are less sensitive than the parental leukaemia (solid line) to Compound E treatment. **c,** The additive effect of combining GDC-0941 with 0.1 μM Compound E (dashed line) in the parental leukaemia (P) is absent in resistant leukaemias 2M R1 and 2M R3. **d,** Resistant leukaemias with insertions in *Aph1a* (2M R1) and *Notch1* (JW81 R5) have markedly lower NICD and Myc protein levels than the parental leukaemias (P).

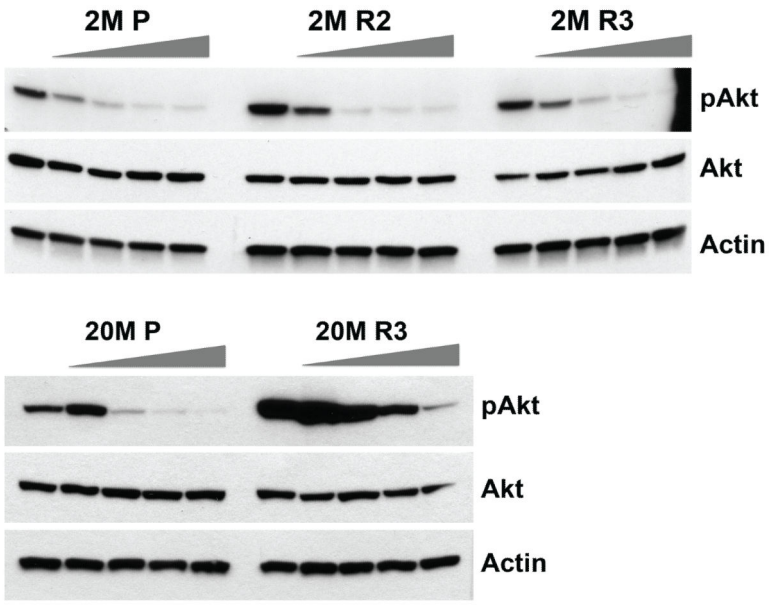

Figure ED6. Increasing the concentration of GDC-0941 inhibits Akt phosphorylation in GDC-0941-resistant T-ALL

Primary drug-resistant leukaemia cells were harvested from recipient mice and exposed to a range of GDC-0941 doses for 30 min (triangles; dose range 0-5.0 μM). Western blotting reveals higher basal levels of pAkt S473 in resistant leukaemias compared to the corresponding parental T-ALL. Higher doses of GDC-0941 were required to suppress pAkt levels in the resistant leukaemias.

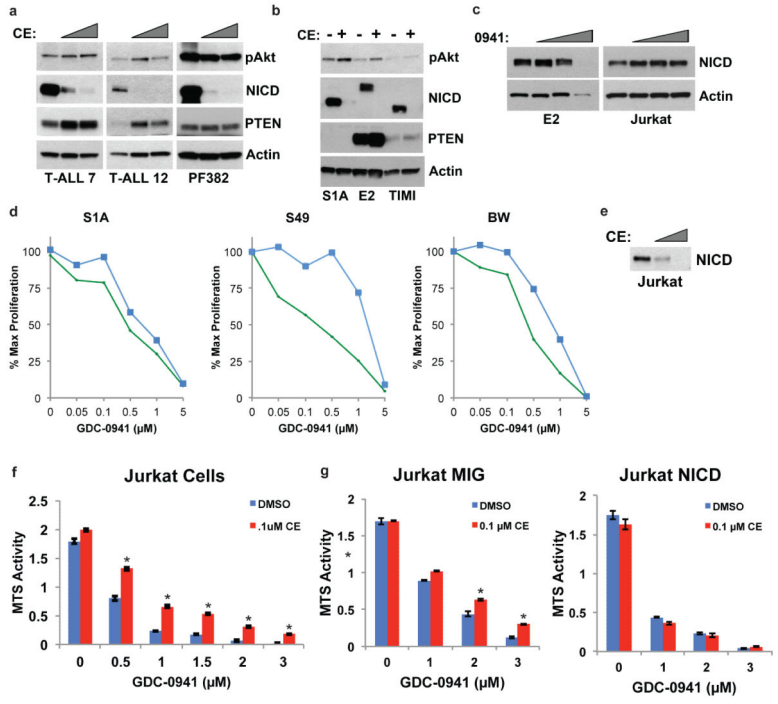

Figure ED7. Notch1 modulates sensitivity to GDC-0941 in T-ALL cell lines

a,b Exposing PTEN positive cell lines T-ALL 7, T-ALL 12, PF382 to 0.1 & 1 μM of Compound E (panel **a**) and lines E2 and TIMI to 1 μM (panel **b**) consistently reduced (01. μM concentration) or eliminated (1 μM concentration) NICD expression and had variable effects on PTEN expression and pAkt S473 levels as assessed by Western Blotting. **c,** E2 and Jurkat T-ALL cell lines were treated with GDC-0941 (0941; triangles, .01, 0.1, and 1 μM) for 24 hr. Western blotting indicates that GDC-0941 does not inhibit affect NICD levels. The 1 μM dose of GDC-0941 induces extensive cell death in E2 cells as indicated by reduced Actin. **d,** S1A, S49 and BW cells were transduced with a construct co-expressing NICD and GFP. Transduced cells were plated in varying doses of GDC-0941. After 3 days, numbers of viable GFP^+ (green line) and GFP^- cells (blue line) were counted by flow cytometry and equalized to DMSO control (% Max). **e,** Exposing Jurkat cells to 0.1 or 1.0 μM Compound E for 24 hr resulted in a dose dependent reduction in NICD protein levels. **f,** Jurkat cells were exposed to a range of GDC-0941 concentrations with 0.1 μM Compound E or control vehicle (DMSO) for 72 hr and proliferation was measured by MTS assay. The addition of Compound E significantly reduced the efficacy of GDC-0941 at multiple concentrations. **g,** Stable Jurkat clones expressing either control vector (MIG) or NICD were treated with GDC-0941 and Compound E according to the same protocol in panel **f**. Note that enforced expression of NICD rescues the Compound E-mediated growth inhibition. **f, g,** Error bars show S.E.M. of 3 technical replicates. Asterisks indicate significant differences (2-sided t test, p values < .05).

Extended Data Table 1

Difference in median survival between drug-treated and vehicle- treated mice for 21 primary T-ALLs. Individual T-ALLs were classified as responsive to GDC- 0941 or the GDC-0941/ PD901 combination if treatment extended median survival by > 3 days in recipient mice. We performed statistical analysis (Log-rank test) of each T-ALL to compare the survival of recipients treated with the control vehicle or drug(s). P values are shown in parenthesis in the respective columns. Note that T-ALLs with a statistically significant difference between treated and control mice of less than 3 days were classified as resistant (e.g. 78M). By contrast, treating T-ALLs 10M and 15M with the GDC-0941/PD901 combination resulted in a substantial survival benefit that did not reach statistical significance. These leukemias (*) were classified as sensitive to treatment based on the markedly extended survivals in treated animals.

GDC-0941 Responsive T-ALLs

T-ALL	*Kras* genotype	*Notch1* genotype	Vehicle Survival (days)	GDC-0941 Survival Days (P value)	GDC-0941 + PD901 Survival Days (P value)
2M	G12D	Mutant	27.5	37 (.02)	77 (<.0001)
4585	G12D	Mutant	9	14.5 (.05)	N/D
5B	G12D	Mutant	12	16 (.02)	28 (.005)
20M	G12D	Mutant	17	21 (.03)	33 (.004)
JW-81	WT	Mutant	15	25 (.008)	35 (.008)
JW-14	WT	N/A	10	19 (.005)	30 (.005)
5C	WT	WT	18	22 (.004)	N/D

GDC-0941/PD0325901 Responsive T-ALLs

T-ALL	*Kras* genotype	*Notch1* genotype	Vehicle Survival (days)	GDC-0941 Survival Days (P value)	GDC-0941 + PD901 Survival Days (P value)
73A	G12D	WT	17	24 (.2)	37 (.005)
10M	G12D	Mutant	16	19 (.5)	23* (.06)
7M	G12D	Mutant	13	15 (.007)	31 (.007)
9M	G12D	Mutant	15	16 (.5)	25 (.005)
4B	G12D	Mutant	23	21.5 (.8)	36.5 (.01)
15M	G12D	Mutant	22	18 (.5)	47* (.08)
8633	WT	Mutant	15	15(.4)	24 (.008)

Non-Responsive T-ALLs

T-ALL	*Kras* genotype	*Notch1* genotype	Vehicle Survival (days)	GDC-0941 Survival Days (P value)	GDC-0941 + PD901 Survival Days (P value)
78M	G12D	Mutant	8	9 (.008)	N/D
12M	G12D	Mutant	11	11 (.9)	13 (.01)
73M	G12D	Mutant	9.5	7 (.03)	10 (.09)
78A	G12D	Mutant	10	9 (.3)	9 (.04)
29B	G12D	Mutant	10	10 (.9)	N/D
4368	WT	WT	30.5	35 (.7)	37.5 (.3)
12-0	WT	WT	15	13 (.7)	16 (.2)

Extended Data Table 2

Profile of retroviral insertion sites in parental leukemias and resistant variants. TOP, Data for 3 different parental and related resistant leukemias (2M P/R1, 8633 P/R1 & JW81 P/R5). Each row corresponds to a single viral integration site. Total (%) number of integrations cloned at each site from each tumor is presented as total and as a percent of total integrations. Genes listed are those within 25 KB or the closest gene within 100 KB. In some cases multiple genes separated by | are listed. Note that the Aph1 and Notch1 insertion was only detected in the resistant leukemia, whereas the Dtx1 insertion was present at very low levels in the parental indicating it was a pre-existing subclone. **Bottom,** Information on Notch1 related genes including location and direction of the retrovirus relative to the gene.

	2M Parental	2M Resistant 1
Gene Symbol(s)	**Total (%)**	**Total (%)**
Ptprt	18 (23)	28 (24)
Sdc1	11 (14)	22 (19)
Tlr5	10 (13)	15 (13)
Ablim1	9 (12)	4 (3)
Cux1	6 (8)	0
Arvcf | Comt	5 (6)	6 (5)
Anp32e | Car14 | **Aph1a**	**0**	**12 (10)**
Trim29	0	7 (6)
Total informative insertions:	n=77	n=117

	8633 Parental	8633 Resistant 1
Gene Symbol(s)	**Total (%)**	**Total (%)**
Olfr56 | Ifi47 | Olfr1396	40 (47)	63 (57)
Msh6 | Fbxo11	12 (14)	0
Slc22a8	4 (5)	5 (5)
Dtx1 | Rasal1	**1 (1)**	**21 (19)**
N/D	0	8 (7)
Slc22a8	0	5 (5)
Alox5ap	0	4 (4)
Total informative insertions:	n=86	n=111

	JW-81 Parental	JW-81 Resistant 5
Gene Symbol(s)	**Total (%)**	**Total (%)**
Cntnap2	16 (15)	1 (1)
Fam55d	13 (12)	9 (9)
N/D	11 (10)	2 (2)
Wasf2	10 (9)	0
Zc3h12d | Ppil4	8 (7)	14 (15)
Rasgrp1	8 (7)	6 (6)
Dusp2 | Astl	6 (6)	6 (6)
Slc43a1 | Timm10 | Smtnl1	5 (5)	3 (3)
Mir17 | Mir18 | Mir19a	5 (5)	3 (3)
Ikzf1	5 (5)	4 (4)
Ifngr1 | Il22ra2	5 (5)	5 (5)
Vmn2r99	0	10 (11)
Notch1	**0**	**4 (4)**
Mir29b-1 | Mir29a	0	4 (4)
Total informative insertions:	n=109	n=95

Gene	Insertion location; orientation	Gene Function
Aph1a	3 prime; inverse	Subunit of the of gamma secretase complex
Dtx1	Intron 3; same	Notch1 target gene. Can act as either a positive regulator or negative regulator of Notch1 depending on developmental and cellular context
Notch1	Intron 3; same	Transmembrane receptor, plays role in controlling cell fate decisions, frequently mutated in T-ALL

Extended Data Table 3

Top, Primary T-ALL samples analyzed in Fig 3b. Bottom, Pairwise comparisons shown in Figure 3b.

Sample ID	Gene Expression Array ID	Parental Leukemia	Sample Description	*Kras* Status
KST001	KST001 -htmg430pm.CEL	8633	Parental	WT
KST002	KST002-htmg430pm.CEL	8633	Resistant-0941	WT
KST005	KST005-htmg430pm.CEL	73A	Parental	G12D
KST006	KST006-htmg430pm.CEL	73A	Parental	G12D
KST007	KST007-htmg430pm.CEL	73A	Resistant-0941	G12D
KST008	KST008-htmg430pm.CEL	73A	Resistant-0941/PD901	G12D
KST010	KST010-htmg430pm.CEL	2M	Parental	G12D
KST013	KST013-htmg430pm.CEL	2M	Resistant-0941	G12D
KST014	KST014-htmg430pm.CEL	2M	Resistant-0941	G12D
KST016	KST016-htmg430pm.CEL	2M	Resistant-0941/PD901	G12D
KST021	KST021 -htmg430pm.CEL	2M	Resistant-0941/PD901	G12D
KST023	KST023-htmg430pm.CEL	10M	Parental	G12D
KST024	KST024-htmg430pm.CEL	10M	Resistant-0941	G12D
KST025	KST025-htmg430pm.CEL	10M	Resistant-0941/PD901	G12D

Sample ID	Gene Expression Array ID	Parental Leukemia	Sample Description	*Kras* Status
KST026	KST026-htmg430pm.CEL	10M	Parental	G12D
KST034	KST034-htmg430pm.CEL	20M	Parental	G12D
KST035	KST035-htmg430pm.CEL	20M	Parental	G12D
KST036	KST036-htmg430pm.CEL	20M	Resistant-0941	G12D
KST038	KST038-htmg430pm.CEL	20M	Resistant-0941	G12D
KST039	KST039-htmg430pm.CEL	20M	Resistant-0941	G12D
KST041	KST041-htmg430pm.CEL	20M	Resistant-0941	G12D
KST046	KST046-htmg430pm.CEL	4B	Parental	G12D
KST051	KST051 -htmg430pm.CEL	4B	Resistant-0941/PD901	G12D
KST052	KST052-htmg430pm.CEL	JW-81	Parental	WT
KST056	KST056-htmg430pm.CEL	JW-81	Resistant-0941/PD901	WT
KST057	KST057-htmg430pm.CEL	JW-81	Resistant-0941/PD901	WT
KST062	KST062-htmg430pm.CEL	5C	Resistant-0941	WT
KST077	KST077-htmg430pm.CEL	5C	Parental	WT

Lane	Parental Sample	Resistant Sample
1	KST001	KST002
2	KST010	KST013
3	KST010	KST021
4	KST034	KST039
5	KST035	KST039
6	KST034	KST038
7	KST035	KST038
8	KST052	KST057
9	KST052	KST056
10	KST005	KST007
11	KST006	KST007
12	KST005	KST008
13	KST006	KST008
14	KST010	KST014
15	KST010	KST016
16	KST034	KST036
17	KST035	KST036
18	KST034	KST041
19	KST035	KST041
20	KST023	KST025
21	KST026	KST025
22	KST046	KST051
23	KST077	KST062

Lane	Parental Sample	Resistant Sample
24	KST023	KST024
25	KST026	KST024

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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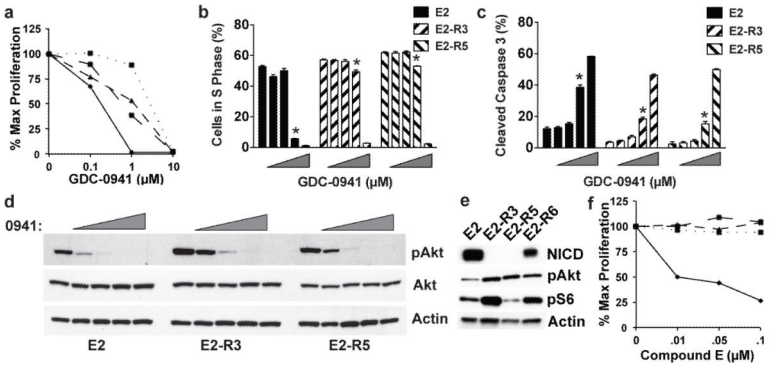

Figure 1. GDC-0941-resistant T-ALL lines down-regulate NICD and increase pAkt

a, GDC-0941 (0941) dose escalation yielded three resistant lines (dotted lines) from T-ALL cell line E2 (solid line) **b-d,** Resistant T-ALL cells were exposed to 0941 (triangles, .01 − 1 μM). Resistant cells require a 10 fold higher dose of 0941 to inhibit BrdU incorporation (**b**) and have impaired cleaved caspase 3 induction (**c**). Error bars reflect S.E.M. of 3 technical replicates with differences between parental and resistant cells at 1 μM marked with an asterisk (2-sided t test, **b,** R3, R5 $p < .0001$; **c,** R3, R5 $p = .0004$) **d,** Western blotting 20 min after GDC-0941 exposure shows that elevated pAkt S473 levels are suppressed in resistant lines by higher drug concentrations. **e,** Activated Notch1 (NICD) is reduced in resistant T-ALL cells, which are also resistant to Compound E (**f;** dotted lines). The experiments in **a-e** were performed 3 times, and in **f** in duplicate.

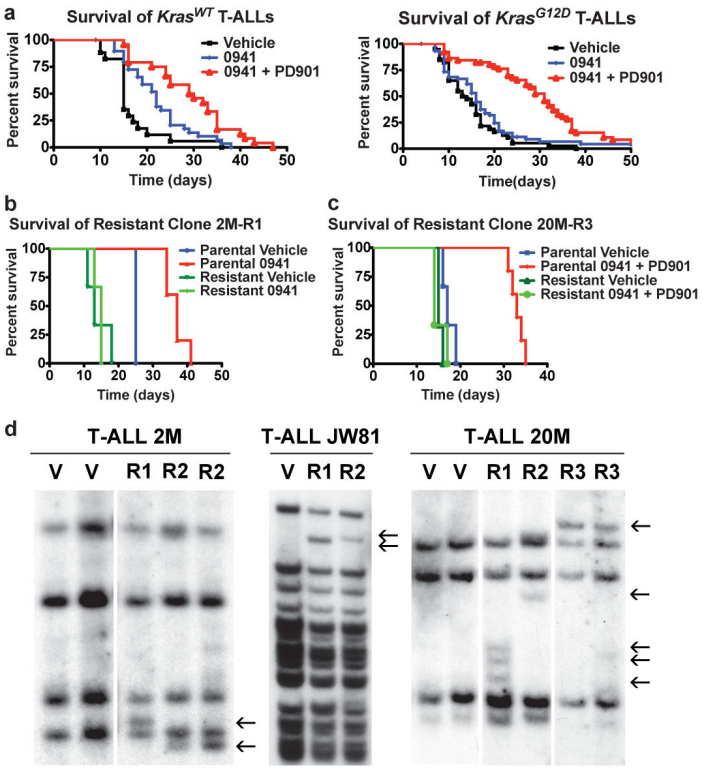

Figure 2. Responses to targeted agents and clonal evolution

a, Kaplan Meier analysis of *Kras*^{WT} T-ALLs treated with vehicle (n = 17), GDC-0941 (0941; n = 29), or 0941/PD901 (n = 24). Treatment with 0941 or the combination extended survival (Log-rank, p = 0.013 and p<.0001). **b,** *Kras*^{G12D} T-ALLs treated with vehicle (n = 39), 0941 (n = 55), or 0941/PD901 (n = 47) only responded to the combination (Log-rank, p<.0001). **c,** Refractory leukaemias have intrinsic drug resistance. Each relapsed T-ALL was transplanted into secondary recipients and retreated with 0941 (2M R1) or 0941/PD901 (20M R3). Relapsed leukaemias (green lines) are unresponsive to treatment that was efficacious in parental T-ALLs (compare blue and red lines) **d,** Southern blot analysis detects novel retroviral integrations (arrows) in resistant leukaemias that are absent in vehicle treated mice (V). Genetically distinct subclones are designated R1, R2, etc.

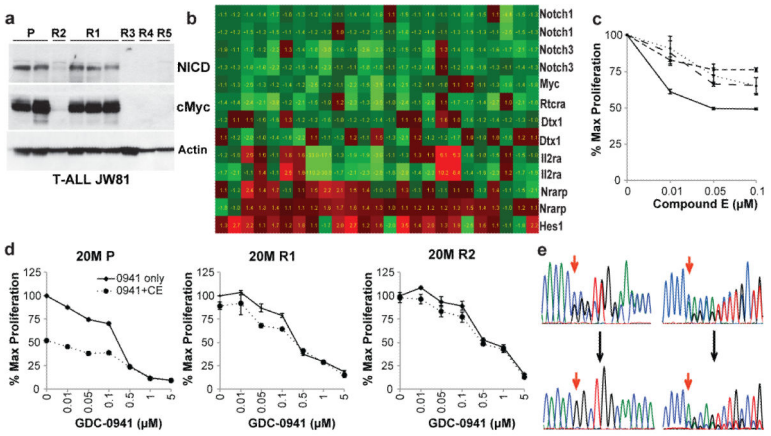

Figure 3. Resistant T-ALLs have impaired Notch1 signalling and are resistant to Compound E

a, Biochemical analysis of resistant clones (R1-R5). Each lane represents an independent sample (n = 2 for JW81, n = 3 for JW81 R1). **b,** Expression profiling of Notch1 target genes in 17 parental/resistant leukaemia pairs (8 in duplicate). **c, d,** T-ALL 20M and resistant clones were exposed to Compound E (panel **c**) or to 0.1 μM Compound E and different GDC-0941 concentrations (panel **d**) for 48 hours. Error bars show S.E.M. of 3 technical replicates. **e,** Notch1 mutations (red arrows) in parental leukaemias (top) are absent or dramatically reduced in resistant leukaemias (bottom).

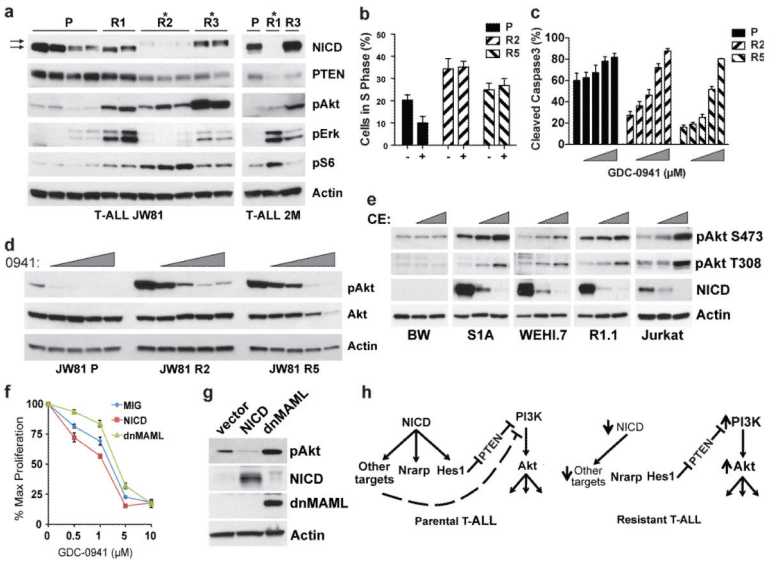

Figure 4. Notch1 modulates PI3K signalling and GDC-0941 (0941) sensitivity

a, Western blot of JW81 and 2M and drug-resistant clones. Asterisks mark resistant T-ALLs with altered Notch1 signalling. Resistant T-ALLs JW81 R2 and R3 lack the *Notch1* mutation in JW81 and thus express a larger Notch protein (upper arrow). Resistant T-ALLs have increased pAkt S473 levels and 4/5 show reduced PTEN expression. **b,** BrdU incorporation is reduced in parental T-ALL cells upon *in vivo* treatment (asterisk, p = .004, 2-sided t test). **c,** Basal apoptosis levels are higher in parental T-ALLs exposed to 0941 *ex vivo* (asterisk, p = .003, 2-sided t test) and are induced after drug exposure in resistant leukaemias. **b, c,** Error bars show S.E.M. of ≥ 3 mice. **d,** pAkt S473 levels in T-ALL cells 20 min after 0941 exposure. **e,** Dose-dependent decrease in NICD levels in T-ALL lines cultured with Compound E for 72 hours correlates with increased pAkt (3 independent experiments). **f,** NICD expression enhances 0941 sensitivity in Jurkat cells, while dominant negative Mastermind (dnMAML) reduces sensitivity compared to control vector (MIG). Error bars show S.E.M. of 3 experiments. **g,** Western blot showing NICD, dnMAML, and pAkt S473 in Jurkat cells from panel **f**. **h,** Model of Notch1-PI3K crosstalk in sensitive and resistant T-ALLs (see text).