Mutant *Ikzf1*, *Kras*^{G12D}, and *Notch1* cooperate in T lineage leukemogenesis and modulate responses to targeted agents

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Mice that accurately model the genetic diversity found in human cancer are valuable tools for interrogating disease mechanisms and investigating novel therapeutic strategies. We performed insertional mutagenesis with the MOL4070LTR retrovirus in *Mx1-Cre, Kras*^{G12D} mice and generated a large cohort of T lineage acute lymphoblastic leukemias (T-ALLs). Molecular analysis infers that retroviral integration within *Ikzf1* is an early event in leukemogenesis that precedes *Kras*^{G12D} expression and later acquisition of somatic *Notch1* mutations. Importantly, biochemical analysis uncovered unexpected heterogeneity, which suggests that Ras signaling networks are remodeled during multistep tumorigenesis. We tested tumor-derived cell lines to identify biomarkers of therapeutic response to targeted inhibitors. Whereas all T-ALLs tested were sensitive to a dual-specificity phosphoinositol 3-kinase/mammalian target of rapamycin inhibitor, biochemical evidence of Notch1 activation correlated with sensitivity to γ-secretase inhibition. In addition, *Kras*^{G12D} T-ALLs were more responsive to a MAP/ERK kinase inhibitor in vitro and in vivo. Together, these studies identify a genetic pathway involving *Ikzf1*, *Kras*^{G12D}, and *Notch1* in T lineage leukemogenesis, reveal unexpected diversity in Ras-regulated signaling networks, and define biomarkers of drug responses that may inform treatment strategies.

K-ras | retroviral insertional mutagenesis | T cell leukemia | targeted therapeutics | Ikaros

T lineage acute lymphoblastic leukemia (T-ALL) is characterized by aberrant clonal proliferation and tissue invasion of lymphoblasts (reviewed in ref. 1). Although intensive treatment protocols have markedly improved the outcomes of children and adolescents with T-ALL, cure rates for adults remain below 50%, and the prognosis is poor for patients who relapse at any age (1, 2). Modern treatment regimens also carry a substantial risk of adverse late effects (3). Thus, the development of more effective and less toxic therapies that are based on the underlying molecular lesions is a high priority.

Recent studies have advanced our understanding of T-ALL pathogenesis (reviewed in refs. 4–6). Genes encoding transcription factors, such as *TAL1, LYL1, LMO1*, and *LMO2*, are frequently deregulated by chromosomal translocations in T-ALL (7). *NOTCH1* was first implicated in leukemogenesis through a t (7;9) chromosomal translocation that truncates and constitutively activates the Notch1 protein (8), and gain-of-function *NOTCH1* mutations within the heterodimerization (HD) and/or proline-, glutamic acid-, serine-, and threonine-rich (PEST) domains are found in ≈55% of primary human T-ALL specimens (9). Emerging data also support an important role for aberrant Ras signaling in T-ALL. *NRAS* and *KRAS2* mutations are found in 10–15% of cases (10, 11), whereas the *NF1* tumor suppressor gene is inactivated in ≈3% (12). Chromosomal translocations that result in fusions of *ABL* and *JAK2*, kinases that are known to activate Ras, are also found in T-ALL (11). More recently, somatic gain-of-function *JAK1* mutations were discovered in 18–27% of adult and in 2% of pediatric T-ALL cases, respectively (13, 14). These leukemias demonstrated elevated levels of phosphorylated ERK and Akt, which are important effectors of activated Ras. The *PTEN* tumor suppressor, which encodes a lipid phosphatase that negatively regulates the phosphoinositol 3-kinase (PI3K)/Akt signaling pathway, is mutated in 5–8% of T-ALLs, and reduced expression was observed in an additional 17% of cases (15, 16). Recent studies that uncovered PI3K pathway mutations in ≈50% of pediatric T-ALLs underscore the central role of this Ras effector cascade in leukemic growth (17–19).

Observations in mice further implicate hyperactive Ras in T-ALL pathogenesis. Transgenic mice overexpressing *Nras* or *Rasgrp1* develop T lineage lymphomas (20, 21). In addition, thymic lymphomas are observed in ≈30% of mice harboring a latent oncogenic *Kras*^{G12D} allele that is activated by spontaneous recombination (22). Furthermore, the observation that most of these mice do not develop T-ALL infers that additional mutations are required. Using the IFN-regulated *Mx1-Cre* transgene to activate a conditional mutant *Kras*$^{LSL\text{-}G12D}$ allele in hematopoietic cells causes an aggressive myeloproliferative disease (MPD) (23, 24). Interestingly, transferring bone marrow from these mice into irradiated recipients results in T-ALL (25, 26), and limit dilution experiments showed that one to three *Kras* mutant hematopoietic stem cells were sufficient to initiate T-ALL in vivo (26, 27). Consistent with data from other murine T-ALL models, these leukemias acquired somatic *Notch1* mutations (4, 25, 26, 28).

Retroviral insertional mutagenesis (RIM) in mice is a robust strategy that has been used to identify genes that are involved in human leukemia, including *Ikzf1* (Ikaros), *Notch1*, and *Lmo2* in T-ALL (29–32). We used the MOL4070LTR retrovirus (33) to perform RIM in *Mx1-Cre, Kras*^{G12D} mice. Here we show that aberrant Ikaros expression due to viral integrations is a frequent early event and that somatic *Notch1* mutations arise later and cooperate with oncogenic *Kras* in leukemogenesis. We generated a large panel of tumor-derived cell lines for biochemical and preclinical studies. Analysis of Ras and Notch1 signaling uncovered unexpected heterogeneity in T-ALL cell lines and in primary leukemias. Exposing T-ALL cells to targeted agents in vitro and in vivo uncovered markers of drug response and revealed synergistic effects of γ-secretase with MAP/ERK kinase (MEK) or PI3K/mammalian target of rapamycin (mTOR) inhibitors. These data demonstrate the value of using diverse panels of related cancers for identifying and ordering mutations,

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interrogating cancer signaling networks, and discovering molecular markers of drug sensitivity.

Results

MOL4070LTR Induces T-ALL in *Mx1-Cre, Kras$^{LSL-G12D}$* Mice. We injected neonatal *Mx1-Cre, Kras$^{LSL-G12D}$* mice (*Kras^{G12D}*) and wild-type littermates (*Kras^{WT}*) with MOL4070LTR to induce diverse retroviral integrations within the expanding hematopoietic compartment. *Kras^{G12D}* expression was then activated at 3 weeks of age by administering a single dose of polyinosinic-polycytidilic acid (pIpC). All *Kras^{G12D}* mice that were infected with MOL4070LTR developed MPD without overt evidence of acute leukemia. We reasoned that the rapid progression of the MPD might provide insufficient time for retrovirally induced hematologic malignancies to emerge. To test this idea, we exploited the fact that the *Kras^{G12D}*-induced MPD is not transplantable into sublethally irradiated mice (23, 24) and transferred bone marrow cells from 48 moribund *Kras^{G12D}* mice into 3–5 recipients that received 450 cGy of radiation (Fig. 1*A*). We refer to bone marrow donors with MPD as "virus-injected mice" throughout this article. Twenty bone marrows from virus-injected mice (42%) induced T-ALL, and nine others (19%) resulted in transplantable myeloid malignancies (Fig. 1*A* and Fig. S1). By contrast, the frequencies of T-ALL and myeloid malignancies in *Kras^{WT}* littermates that received MOL4070LTR and were observed for ≈15 months were 21% and 51%, respectively (Fig. 1*A*). Latency was determined by adding the time to death from MPD in the virus-injected mice and the time to the development of a myeloid malignancy (Fig. 1*B*) or T-ALL (Fig. 1*C*) in transplant recipients. *Kras^{G12D}* expression reduced acute myeloid leukemia latency from 336 to 122 days ($P < 0.0001$; Fig. 1*B*) and T-ALL latency from 226 to 151 days ($P < 0.001$; Fig. 1*C*).

Primary recipients with T-ALL showed a large thymic mass, modest leukocytosis with lymphoblasts visible on blood smears, and extensive infiltration of multiple tissues (Fig. S2). T-ALL cells of thymic origin readily induced T-ALL in secondary recipients (Fig. 1*D*). *Kras^{G12D}* and *Kras^{WT}* T-ALLs from primary and secondary recipient mice are arrested at an immature stage of development, and most express CD4 and CD8. Southern blot analysis of primary T-ALLs revealed a clonal integration pattern that was not detected in the marrows of donor virus-injected mice (Fig. 1 *D* and *E*). Interestingly, a single donor bone marrow could initiate independent T-ALLs in different primary recipients (Fig. 1 *D* and *E*), which invariably demonstrated a stable pattern of retroviral insertions in secondary transplants (Fig. 1 *D* and *F*). Secondary recipients developed aggressive leukemia with reduced latency, bone marrow effacement, and variable infiltration of the thymus.

MOL4070LTR Integrations Disrupt the *Ikzf1* Locus in *Kras^{G12D}* T-ALLs. We cloned retroviral integration sites from 24 *Kras^{G12D}* T-ALLs and 6 *Kras^{WT}* T-ALLs (Table S1). The criteria used to define common insertion sites (CIS) were (*i*) genes must be identified in at least two independent tumors; (*ii*) an individual integration must be recovered more than five times (of 192 independent sequenced clones per T-ALL); and (*iii*) one integration must be within 30 kb upstream/downstream of the identified gene. *Ikzf1* (Ikaros) was the most frequent CIS ($n = 9$) and was restricted to *Kras^{G12D}* tumors. All nine integrations were within the *Ikzf1* gene, and most were predicted to disrupt Ikaros function (Table S1). Consistent with this, RT-PCR analysis of leukemias with *Ikzf1* integrations revealed aberrant cDNA expression. Western blotting demonstrated absence of the high-molecular-weight Ikaros protein, which is associated with full biologic activity, and expression of truncated dominant negative Ikaros isoforms (Fig. S3*A*). Interestingly, whereas *Kras^{G12D}* T-ALLs without *Ikzf1* insertions showed robust expression of the high-molecular-weight form of Ikaros ($n = 3$), thymus lysates from *Kras^{G12D}* mice without T-ALL unexpectedly showed reduced expression of this isoform (Fig. S3*A*). We performed a retroviral transduction/transplantation experiment to assess the effect of expressing a dominant negative Ikaros isoform in *Kras* mutant bone marrow cells (32, 34). Recipient mice that were transplanted with these cells developed T-ALL (Fig. S3*B*).

We identified three other CIS including *Rasgrp1* integrations, which occurred in three of six of the *Kras^{WT}* tumors and are likely to be functionally redundant with *Kras^{G12D}* expression (Table S1).

Somatic *Notch1* Mutations in MOL4070LTR-Induced T-ALLs. Western blotting with an antibody that detects the Notch1 intracellular domain (NICD) revealed evidence of pathway activation in 90% of primary *Kras^{G12D}* T-ALLs (Fig. 2*A*), and sequence analysis identified truncating PEST domain mutations that are similar to those observed in human T-ALL (Table S2). We detected *Notch1* mutations in all 30 *Kras^{G12D}* T-ALLs examined. By contrast, only 3 of 9 *Kras^{WT}* T-ALLs (33%) contained *Notch1* mutations. Similarly, all 11 *Kras^{G12D}* T-ALL cell lines shown in Table 1 carried *Notch1* mutations, compared with 1 of 5 *Kras^{WT}* lines. As expected, each cell line harbored the same mutation as the parental T-ALL. Interestingly, however, we observed variable levels of NICD in T-ALL cell lines with *Notch1* mutations, including a number in which NICD was not visualized by Western blotting (Fig. 2*B*, lanes 12, 13, 15, and 17, and Table 1).

To further assess the relationship of *Notch1* mutations and NICD protein expression to Notch pathway activation, we used quantitative real-time PCR to assay the Notch target genes *Deltex1* and *Hes1* in seven T-ALL cell lines (Fig. 2*C*). Elevated *Deltex1* expression was observed in two cell lines in which NICD

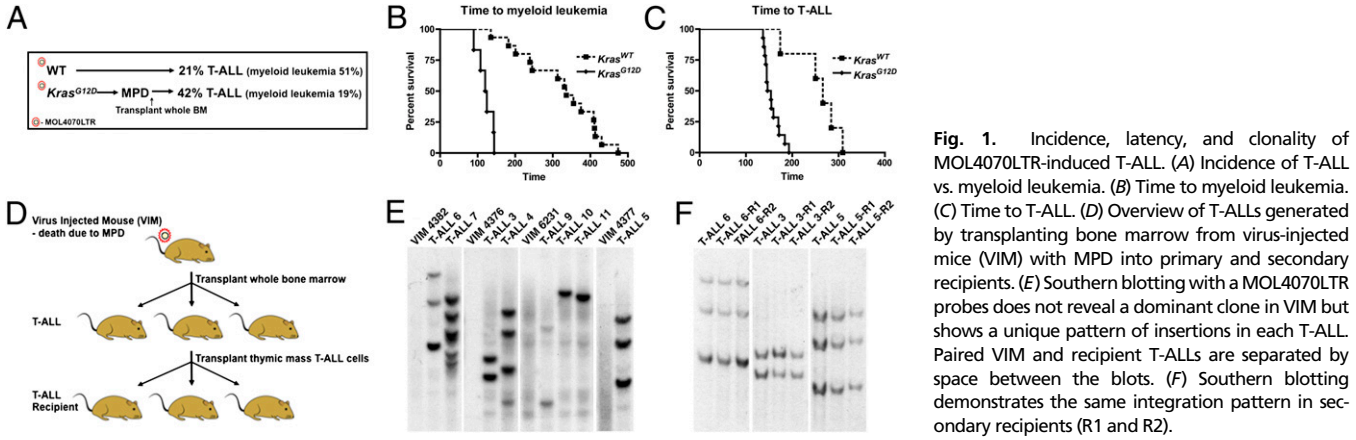

Fig. 1. Incidence, latency, and clonality of MOL4070LTR-induced T-ALL. (*A*) Incidence of T-ALL vs. myeloid leukemia. (*B*) Time to myeloid leukemia. (*C*) Time to T-ALL. (*D*) Overview of T-ALLs generated by transplanting bone marrow from virus-injected mice (VIM) with MPD into primary and secondary recipients. (*E*) Southern blotting with a MOL4070LTR probes does not reveal a dominant clone in VIM but shows a unique pattern of insertions in each T-ALL. Paired VIM and recipient T-ALLs are separated by space between the blots. (*F*) Southern blotting demonstrates the same integration pattern in secondary recipients (R1 and R2).

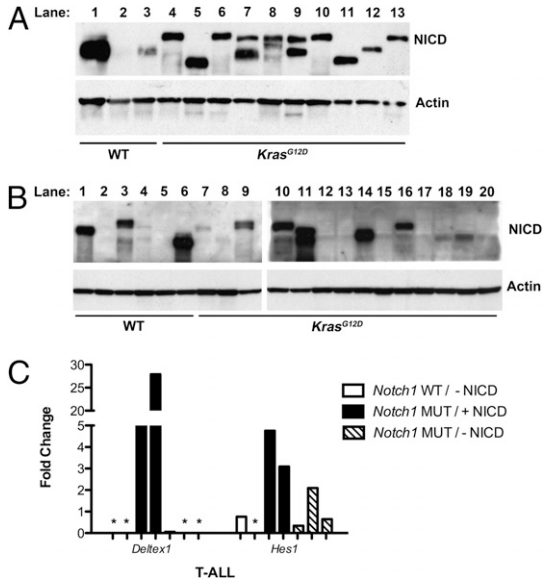

Fig. 2. Notch1 is deregulated in MOL4070LTR-induced T-ALL. Western blot analysis of NICD expression in (*A*) primary T-ALLs and (*B*) T-ALL cell lines. (*C*) Expression of *Deltex1* and *Hes1* were assessed by quantitative RT-PCR in cell lines without a *Notch1* mutation or detectable NICD by Western blotting (*Notch1* WT/-NICD; $n = 2$); with a *Notch1* mutation and detectable NICD (*Notch1* MUT/+NICD; $n = 2$); and with a *Notch1* mutation but not detectable NICD (*Notch1* MUT/-NICD; $n = 3$). Asterisks (*) indicate undetectable levels.

was visualized by Western blotting but not in three lines with *Notch1* mutations that lacked NICD (Fig. 2*C*). As expected, *Deltex1* expression was not detected in two T-ALL cell lines without *Notch1* mutations (Fig. 2*C*). Although less dramatic, *Hes1* expression demonstrated a similar overall pattern, with the highest levels observed in the two cell lines with detectable NICD (Fig. 2*C*). Together, these data support the idea that NICD is a biochemical marker of Notch pathway activation in T-ALL cell lines.

The coexistence of retroviral integrations and *Notch1* mutations in individual T-ALLs allowed us to ascertain the likely order in which these genetic changes were acquired. The outcome shown in Fig. 1 *D* and *E*, in which a single virus-injected $Kras^{G12D}$ bone marrow gave rise to genetically distinct T-ALLs, was especially informative. In these cases, each T-ALL carried a different *Notch1* mutation. Further analysis using allele-specific PCR did not detect *Notch1* mutations in the bone marrows of virus-injected mice (Fig. S4). On the basis of the sensitivity of these assays, it is formally possible that ≈200 or fewer cells of the 2×10^6 that were transplanted could have harbored a *Notch1* mutation. Together, these studies provide strong evidence for sequential acquisition of *Ikzf1*, *Kras*, and *Notch1* mutations during leukemogenesis and support the idea that *Notch1* mutations are acquired after transplantation.

Signaling in T-ALL Cell Lines. We interrogated Ras signaling in cell lines generated from $Kras^{WT}$ and $Kras^{G12D}$ T-ALLs (Table 1). K-Ras^{G12D} accumulates in the active, GTP-bound conformation and exerts its oncogenic effects, in part, by deregulating the Raf1/MEK/ERK and PI3K/Akt/mTOR/S6 effector pathways. $Kras^{G12D}$ T-ALL cell lines uniformly showed markedly elevated levels of Ras-GTP compared with lines generated from $Kras^{WT}$ leukemias (Fig. 3*A*). However, the levels of phosphatase and tensin homolog (PTEN) and of phosphorylated ERK, Akt, and S6 (p-ERK, p-Akt, and p-S6) were highly variable in cell lines of both *Kras* genotypes, and changes in "upstream" pathway components were not highly correlated with downstream proteins. For example, absent or reduced PTEN expression was not uniformly associated with increased p-Akt and p-S6 levels (Fig. 3*B*). Western blot analysis also revealed variable levels of p-ERK, p-Akt, p-S6, and PTEN in primary T-ALLs (Fig. 3*C*). By contrast, $Kras^{G12D}$ expression in untransformed primary thymocytes consistently induced ERK and S6 activation (Fig. 3*C*). The striking biochemical heterogeneity in $Kras^{G12D}$ T-ALLs indicates that oncogenic K-Ras^{G12D} signaling is modulated in the context of multistep tumorigenesis.

Sensitivity of $Kras^{G12D}$ and $Kras^{WT}$ T-ALL Cell Lines to Chemical Inhibitors. Notch1 activation requires cleavage by the γ-secretase protease. Importantly, because many T-ALL–associated Notch1 mutant proteins remain dependent on γ-secretase for biologic activity, treatment with γ-secretase inhibitors (GSIs) is a rational therapeutic strategy for T-ALL (4). We investigated the effects of Compound E, a potent GSI, on the proliferation of T-ALL cell lines. These studies revealed variable sensitivity, which correlated poorly with the presence of a mutation in either *Notch1* or *Kras* (Table 1). However, 10 cell lines in which NICD was detected by Western blotting were sensitive to Compound E, whereas none of 6 lines without visible NICD were sensitive (Table 1, Fig. 4*A*, and Fig. S5).

We also evaluated the effects of the dual-specificity PI3K/mTOR inhibitor PI-103 (35) on the proliferation of T-ALL cell lines and found that all were sensitive to 1 μM of this drug, irrespective of *Kras* genotype or pathway activation as deter-

Table 1. Characteristics of T-ALL cell lines

Primary tumor	Cell line ID	$KRas^{G12D}$ genotype	PD0325901 sensitivity	*Notch1* mutation	NICD detected	GSI sensitivity	Ikaros status
7	7	+	+++	+	++	+	WT
9	9A	+	+++	+	++	+++	DomNeg
12	12	+	+++	+	++	+	Null
6	6	+	+++	+	+	+++	DomNeg
9	9B	+	+++	+	++	+++	DomNeg
17	17	+	++	+	+	+	Null
15	15	+	+++	+	+	++	DomNeg
3	3B	+	+++	+	—	—	WT
29	29	+	+++	+	—	—	WT
3	3A	+	+++	+	—	—	DomNeg
22	22	+	++	+	—	—	DomNeg
C1	C1	—	+	+	+++	++	Null
C3	C3	—	—	—	+	++	DomNeg
C7	C7	—	+	—	—	—	WT
C2	C2	—	—	—	++	+++	Null
^{11}C	^{11}C	—	—	—	—	—	WT

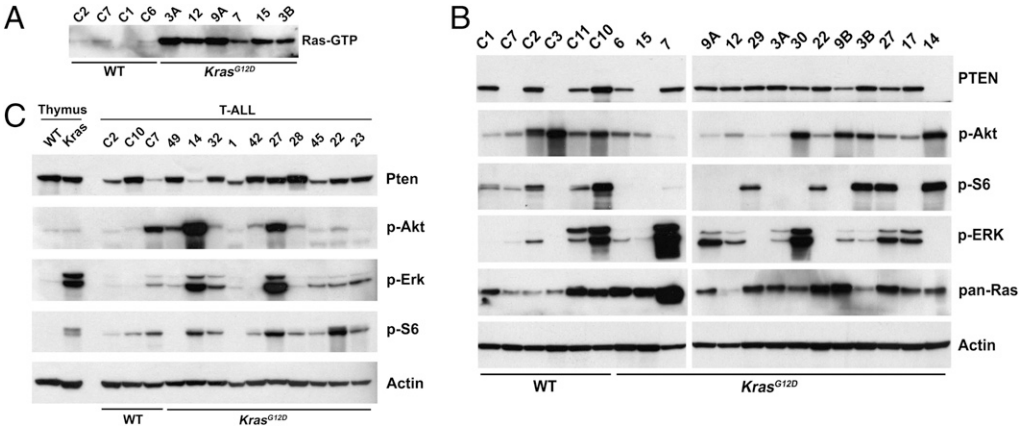

Fig. 3. Ras signaling in T-ALL. (*A*) Ras-GTP levels are elevated in $Kras^{G12D}$ cell lines deprived of serum for 16 h. (*B* and *C*) PTEN p-Akt, p-S6, and p-ERK levels in cell lines under normal growth conditions (*B*) and in primary T-ALLs (*C*).

mined by Western blotting (Figs. 3*B* and 4*B*, Table 1, and Fig. S6 *A* and *B*). By contrast, there was substantial variability in the responses of T-ALL cell lines to the MEK inhibitor PD0325901. Interestingly, $Kras^{G12D}$ T-ALL cell lines showed enhanced sensitivity to PD0325901, which was independent of p-ERK levels (Figs. 3*B* and 4*C*, Table 1, and Fig. S6 *C* and *D*).

Most cell lines that were sensitive to Compound E were also sensitive to PD0325901 (Table 1). We therefore examined whether either inhibitor influences both targets. However, PD325901 had no effect on cleaved Notch1 levels, and Compound E did not reduce p-ERK levels (Fig. S7). The finding that many of the same cell lines were sensitive to PD0325901 and Compound E suggested that blocking both pathways might lead to an enhanced therapeutic effect. Indeed, there is an additive effect on proliferation when these inhibitors are combined at low doses (Fig. 4*D* and Fig. S8*A*). Similarly, exposing cells lines to both PI-103 and Compound E increased the inhibitory effects of either drug alone (Fig. 4*D* and Fig. S8*B*).

PD0325901 Treatment of Primary T-ALLs. To test the prediction that *Kras* genotype might influence MEK dependence in vivo, we established cohorts of eight recipient mice that were transplanted with each of six primary $Kras^{G12D}$ or three $Kras^{WT}$ T-ALLs ($n = 72$ total recipients). Five mice in each group were randomly assigned to treatment with PD0325901, and three received control vehicle. The mice were treated for 4 weeks or until death. Consistent with data from the T-ALL cell lines, therapeutic response to MEK inhibition was associated with the $Kras^{G12D}$ genotype (Fig. 4*E*). Whereas treatment with PD0325901 was associated with a significant increase in median survival in recipients that were injected with $Kras^{G12D}$ T-ALLs ($P > 0.0005$), this inhibitor had no beneficial effect on the $Kras^{WT}$ T-ALLs (Fig. 4*E*). As in human cancer, there was considerable variability among individual T-ALLs with respect to aggressiveness and degree and duration of response.

Discussion

We dissected the molecular events involved in T-ALL pathogenesis and tested potential therapeutic strategies in a diverse panel of T-ALLs generated by RIM. Ikaros is essential for lymphoid development and is mutated or aberrantly expressed in human T and B lineage ALL (36, 37). Our data implicating aberrant Ikaros expression as an initiating event in T lineage leukemia are consistent with previous studies in genetically engineered mice (38). Beverly and Copabianco (32) performed a RIM screen in transgenic mice that express a potent NICD allele (N^{IC} mice) and recovered *Ikzf1* as a CIS. They also showed that expressing mutant Ikaros proteins in N^{IC} bone marrow rapidly induced T-ALL in irradiated recipients (32). Subsequent studies also revealed somatic *Notch1* PEST domain mutations in Ikaros transgenic mice (39, 40).

In contrast to previous RIM studies (30, 41), we found that Notch1 activation invariably resulted from PEST domain mutations rather than from retroviral integrations within the *Notch1* locus. A study in which Chiang et al. (28) used a transduction/transplantation system to compare a series of *Notch1* mutant alleles suggests an explanation for this finding. These authors showed that truncated Notch1 proteins generated as a result of chromosomal translocations or retroviral integrations within the *Notch1* gene are much more potent than PEST domain mutations. These data raise the possibility that truncated *Notch1* alleles may be initiating or early events in T-ALL, whereas biologically "weaker" HD and PEST domain mutations may be obligate cooperating events (4, 28). Our data support this hypothesis. We identified *Ikzf1* disruption as an early event that cooperates strongly with subsequent oncogenic *Kras* expression in T lineage leukemogenesis (Fig. 5*A*). Furthermore, RT-PCR analysis of independent T-ALLs that emerged from the same virus-injected mouse provided strong evidence that *Notch1* mutations are acquired after transplantation (Fig. 5*A*). On the basis of these results, we hypothesize that antecedent *Ikzf1* and *Kras* mutations create a field of thymocytes with aberrant proliferation, survival, and/or differentiation that only require a *Notch1* PEST domain mutation to induce full leukemic transformation.

The combinatorial effects of *Ikzf1*, $Kras^{G12D}$, and *Notch1* mutations on cellular signaling networks are unknown, and molecular markers of responses to targeted agents have not been defined. Whereas K-Ras^{G12D} protein expression resulted in markedly elevated Ras-GTP levels, we unexpectedly observed great variability in the activation of downstream effectors. It is possible that T lymphoid cells adapt to the stress of oncogenic K-Ras expression by modulating effector pathways and that cooperating mutations either antagonize or potentiate this adaptive response. Alternatively, mutations that initiate T-ALL could partially dictate the biochemical consequences of a subsequent *Ras* gene mutation. These potential mechanisms are not mutually exclusive, and our data demonstrate the profound biochemical complexity of primary cancers that arise because of multiple interacting mutations.

Our analysis of a large panel of murine T-ALL cell lines uncovered a complex pattern of GSI response that is consistent with the heterogeneous responses of human T-ALL lines (9). We unexpectedly found a poor correlation between the presence of a *Notch1* mutation and GSI sensitivity and instead identified NICD expression as a highly predictive biomarker of drug response. T-ALL clones that acquire *Notch1* mutations in vivo uniformly express NICD (Fig. 2*A*); however, NICD expression is no longer detected in many tumor-derived cell lines (Fig. 2*B*, lanes 12, 13, 15, and 17). This apparent paradox is not due to widespread clonal infidelity, given that Southern blot analysis of

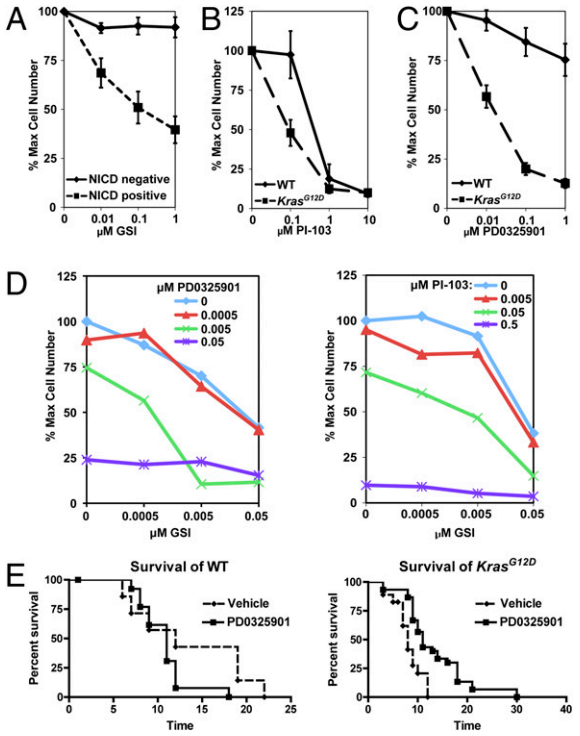

Fig. 4. Effects of small molecule inhibitors on T-ALL cell lines and primary tumors. Cell lines were grown for 5–7 days in the presence of each compound, and the number of viable cells was determined. (*A*) Effects of Compound E, a GSI. The solid line displays the growth of NICD negative cell lines ($n = 6$), and dashed line shows NICD positive lines ($n = 10$; $P = 0.04$–<0.001). (*B*) Effects of PI-103, a dual-specificity PI3K/mTOR inhibitor. $Kras^{WT}$ cell lines are summated in the solid line ($n = 2$), and $Kras^{G12D}$ cell lines are shown as a dashed line ($n = 8$; $P = 0.03$–0.8). (*C*) Effects of PD0325901, a MEK inhibitor. The $Kras^{WT}$ cell lines are shown in the solid line ($n = 5$), and the $Kras^{G12D}$ cells are presented in the dashed line ($n = 11$; $P = 0.0009$–<0.0001). Error bars represent SEM. (*D*) Combined inhibitor treatment. A cell line was grown in increasing concentrations of both (*Left*) GSI and PD0325901 or (*Right*) GSI and PI-103. The blue line (GSI alone) shifts to the left (green and purple lines) when combined with PD0325901 or PI-103, thereby reducing the IC_{50} of GSI. (*E*) PD0325901 prolongs survival in mice transplanted with $Kras^{G12D}$ T-ALLs. Mice were transplanted with three independent primary $Kras^{WT}$ (*Left*) or six independent $Kras^{G12D}$ (*Right*) T-ALLs and were treated with vehicle (dashed line) or PD0325901 (solid line). There was no significant difference in the survival (in days) of mice engrafted with $Kras^{WT}$ leukemias ($P = 0.13$). In contrast, PD0325901 treatment increased survival in recipients of $Kras^{G12D}$ T-ALLs ($P < 0.0005$). $Kras^{WT}$ vehicle $n = 7$, $Kras^{WT}$ PD0325901 $n = 13$, $Kras^{G12D}$ vehicle $n = 15$, and $Kras^{G12D}$ PD0325901 $n = 30$.

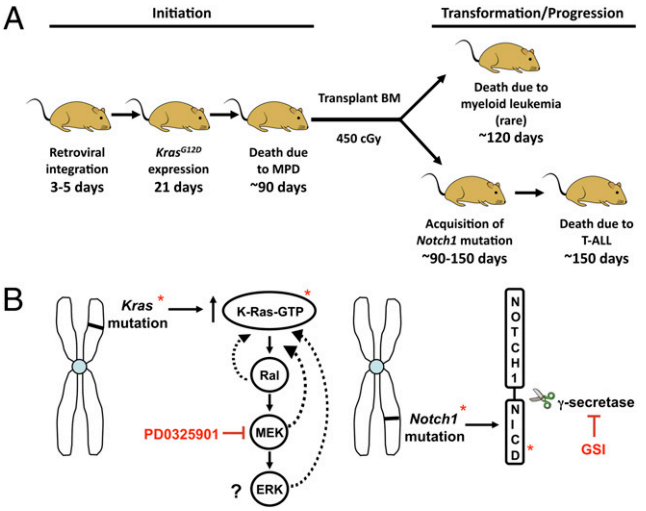

Fig. 5. Models. (*A*) RIM-induced T-ALL in $Kras^{G12D}$ mice. Pups are injected with MOL4070LTR at 3–5 days of age, followed by induction of $Kras^{G12D}$ expression at 21 days. Virus-injected mice (VIM) die of $Kras^{G12D}$-induced MPD at ≈90 days. Bone marrow is then transplanted into sublethally irradiated recipients. Myeloid malignancies develop with short latency. By contrast, an individual VIM can give rise to multiple independent T-ALLs with different retroviral insertion sites and *Notch1* mutations. (*B*) Relationship of MEK and γ-secretase inhibitor responses to mutations found in T-ALL. (*Left*) The pharmacologic target of PD0325901 (MEK) is downstream of oncogenic K-Ras-GTP, which allows for negative feedback and remodeling (dotted lines). As a result, ERK phosphorylation may not accurately reflect pathway activation and/or dependence. (*Right*) *Notch1* PEST domain mutations result in the production of mutant Notch1 proteins that remain dependent on γ-secretase for proteolytic cleavage and biologic activity. NICD levels therefore provide a direct biochemical readout of the deleterious effects of a *Notch1* mutation, and the ability of GSI treatment to reduce these levels is a robust marker of drug response.

paired primary T-ALLs and tumor-derived cell lines revealed the same pattern of MOL4070LTR integrations. A plausible explanation for the loss of NICD expression in many T-ALL cell lines is that aberrant Notch1 signaling is required for leukemic growth in vivo but is dispensable in vitro. If this is true, data from preclinical trials of agents targeting Notch1 signaling that use human and murine cell lines should be interpreted with caution. Our observation that T-ALL cell lines with *Notch1* mutations can survive and proliferate in the absence of NICD expression also suggests outgrowth of a Notch1-indpendent clone as a potential mechanism of acquired GSI resistance.

Despite variable activation of downstream effector pathways in individual T-ALLs, we found that a *Kras* mutant genotype correlated with sensitivity to the MEK inhibitor PD0325901. This is in contrast to our experience with GSI treatment, whereby a biochemical marker (NICD expression) predicted drug sensitivity but the presence of a *Notch1* mutation did not. The relationship between the tumor-associated mutation and the protein targets of PD0325901 and Compound E may explain this difference (Fig. 5*B*). In cancers with oncogenic *Kras* mutations that are treated with a MEK inhibitor, the biochemical target is a downstream component of a signaling cascade, which is subject to extensive feedback regulation that may dramatically alter p-ERK levels. Despite this modulation, our data indicate that the *Kras* mutant T-ALL cells remain dependent on MEK. By contrast, GSIs interfere with the production of a protein that is encoded by mutant *Notch1*. As such, NICD levels provide a biomarker that directly reflects the functional output of the mutant allele. It follows that growth of T-ALL cells that no longer contain NICD would be insensitive to GSI treatment.

Our data have implications for understanding the biology of T-ALL and improving the treatment of this aggressive cancer. Mutations or rearrangements in *KRAS*, *NRAS*, *NF1*, *PTEN*, *PIK3R1*, *PIK3CA*, *AKT*, *ABL*, and *JAK1* occur in ≈60% of T-ALL (10–13, 15–19), and identifying mutations in other signaling proteins may uncover new therapeutic targets. The uniform sensitivity of our diverse panel of cell lines to a PI3K inhibitor is consistent with emerging genetic data implicating deregulated PI3K/PTEN/Akt signaling as playing a pivotal role in T lineage leukemogenesis (15–19). Together, these studies and the observed synergy between Compound E and PI-103 support initiating preclinical and clinical trials of PI3K pathway inhibitors—both alone and in combination with GSIs—in T-ALL.

We have identified NICD expression as a potential biomarker for assessing clinical responsiveness to GSIs. This may be particularly relevant in two contexts. First, human T-ALLs that do not express elevated levels of NICD at diagnosis may not respond to GSI treatment irrespective of *NOTCH1* mutation status. Second, our data support the idea that human T-ALLs with elevated levels of NICD are likely to respond to GSI treatment, and that this will be associated with reduced NICD levels. An intriguing question is whether tumors that subsequently relapse will reactivate NICD expression even in the face of GSI exposure or will acquire the capacity to proliferate

without NICD. Studies of this nature would shed light on potential mechanisms of acquired drug resistance. On the other hand, our observation that tumor genotype (i.e., $Kras^{G12D}$) was a more important determinant of response to the MEK inhibitor PD0325901 than the biochemical readout of p-ERK levels suggests that cancer cells can remain dependent on a specific upstream mutation despite heterogeneous effects on pathway components. These data, in turn, infer that it will be important to compare potential biochemical and genetic biomarkers to predict how cancers will respond to drugs that do not directly target mutant oncoproteins. Insertional mutagenesis is a powerful strategy for approaching the genetic and biochemical complexity of human cancer, for harnessing "chemical genetic" strategies to correlate tumor biology with therapeutic responses, and for generating diverse reagents for preclinical trials of specific agents that can inform human treatment protocols.

Materials and Methods

Mice. All animal experiments conformed to national regulatory standards and were approved by the University of California, San Francisco Committee on Animal Research. C57Bl6/129Sv F1 pups were injected with MOL4070LTR intraperioneally at 3–5 days of age and with pIpC at 21 days of age. For adoptive transfer of acute leukemias, 2×10^6 cells were injected retroorbitally into 8–12 week old wild-type C57Bl6/129Sv F1 recipient mice that had been irradiated with 450 cGy.

Southern Analysis. Genomic DNA was digested with HindIII, followed by electrophoresis and capillary transfer to Hybond-N filters (Amersham). Filters were hybridized with a MOL4070 LTR probe that was labeled with radioactive α-dCTP using Rediprime II (Amersham).

T-ALL Cell Lines. Single-cell suspensions were plated in lymphocyte media (DME-H21, 20% FBS, 10 mM Hepes, penicillin/streptomycin, L-glutamine, 50 μM β-mercaptoethanol, nonessential amino acids, sodium pyruvate, 10 ng/mL IL-2, and 10 ng/mL IL-7). For proliferation assays, $3–5 \times 10^5$ cells were plated per well in a 24-well plate in lymphocyte media plus drug or DMSO (vehicle). After a minimum of four cell doublings in vehicle wells, cells were counted on a Vi-Cell XR (Beckman-Coulter). Cell counts were then normalized to vehicle treated cells.

Preclinical Studies. T-ALL transplant recipients with evidence of leukemia were randomly assigned to receive PD0325901 at a dose of 5 mg/kg per day by oral gavage or a control vehicle. Mice were weighed weekly to adjust the dose and observed daily during treatment (28 days).

Other Methods. Procedures for Shotgun cloning, retroviral transduction and adoptive transfer, Western blotting, Notch1 mutation analysis, and quantitative RT-PCR can be found in *SI Materials and Methods*.

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