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Targeting the NF-κB signaling pathway in Notch1-induced T-cell leukemia

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T-cell acute lymphoblastic leukemia (T-ALL), unlike other ALL types, is only infrequently associated with chromosomal aberrations, but it was recently shown that most individuals with T-ALL carry activating mutations in the *NOTCH1* gene. However, the signaling pathways and target genes responsible for Notch1-induced neoplastic transformation remain undefined. We report here that constitutively active Notch1 activates the NF-κB pathway transcriptionally and via the IκB kinase (IKK) complex, thereby causing increased expression of several well characterized target genes of NF-κB in bone marrow hematopoietic stem cells and progenitors. Our observations demonstrate that the NF-κB pathway is highly active in established human T-ALL and that inhibition of the pathway can efficiently restrict tumor growth both *in vitro* and *in vivo*. These findings identify NF-κB as one of the major mediators of Notch1-induced transformation and suggest that the NF-κB pathway is a potential target of future therapies of T-ALL.

The NF-κB pathway is an important regulator of cell survival, the cell cycle, cell adhesion and migration[1]. As these processes, when deregulated, are the hallmarks of tumor induction, the NF-κB pathway has been implicated in a variety of human cancers[2]. There is also some evidence that NF-κB is involved in the development of T-cell leukemia and lymphoma: it has been demonstrated that T-cell transformation by human T-cell leukemia virus type I involves deregulation of NF-κB signaling[3] and that T cell–specific overexpression of v-Rel leads to T-cell tumors[4]. Moreover, it has recently been shown that deregulated pre–T cell receptor (pre-TCR) signaling results in aberrant activation of the NF-κB pathway that cooperates with transgenic Notch3 to induce T-cell lymphoma[5–7].

Notch1 is a single-transmembrane receptor composed of extracellular (N-EC), transmembrane (N-TM) and intracellular (N-IC) subunits linked via heterodimerization domains[8,9]. Ligand binding induces proteolytic cleavage of the receptor by several proteases (including γ-secretases), resulting in the release of the intracellular fragment of Notch1 (Notch1-IC), which translocates to the nucleus and converts the Cbf1 factor from a transcriptional repressor into an activator. Notch signaling is important in many developmental contexts and is well exemplified by recent studies of Notch1 signaling in the hematopoietic lineage[10]. However, it has been shown that forced expression of Notch1-IC in the hematopoietic lineage of mice is sufficient to induce highly aggressive T-cell leukemia[11–13] and, more importantly, that activating *NOTCH1* mutations are present in most individuals with human T-cell acute lymphoblastic leukemia (T-ALL; refs. 14,15). The *NOTCH1* mutations cluster at the heterodimerization and N-IC domains, and probably result in ligand-independent cleavage and activation of *NOTCH1* and in increased stability of the cleaved, 'active', form of the protein. The identification of *NOTCH1* as the major oncogene in T-ALL opened the way for efforts to elucidate the signaling pathways involved in *NOTCH1*-induced transformation.

We report here that the NF-κB pathway is a major downstream target of Notch1 in T-cell leukemia. We show that constitutively active Notch1 positively regulates the expression of the NF-κB factors Relb and Nfkb2, and activates the IκB kinase (IKK) complex, leading to activation of the NF-κB pathway and increased transcription of several well characterized target genes of NF-κB in lymphocyte progenitors. We also demonstrate that NF-κB activation is important for the induction of T-cell leukemia, as suppression of the NF-κB pathway substantially reduced the severity of the disease. Finally, we show that NF-κB–inhibiting pharmacological agents efficiently induce apoptosis in Notch1-dependent human T-ALL lines. All of these observations strongly suggest that NF-κB activation is an important outcome of Notch1 activity and that targeting NF-κB is a promising strategy for treating T-ALL.

RESULTS

Notch1-induces a T-specific gene profile in BM progenitors

To better understand the molecular mechanism of Notch1-IC–induced T-cell transformation[16,17], we used microarrays and

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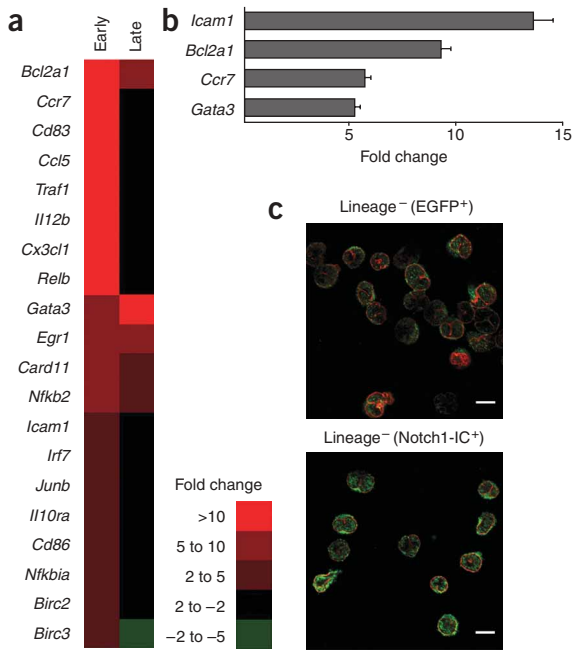

Figure 1 Notch1-IC causes increased expression of target genes of NF-κB in bone marrow hematopoietic progenitors. (**a**) Heat map indicating increased (shades of red) or decreased (shades of green) expression of NF-κB target genes relative to lineage-negative progenitors infected with an EGFP-only retrovirus. The 'early' column represents Notch1-IC–infected lineage-negative progenitors. 'Late' column represents the entire progenitor population (details in **Supplementary Fig. 1**). (**b**) Quantitative RT-PCR analyses of a subset of NF-κB target genes upregulated in the early sample. (**c**) Confocal micrographs of uninfected or Notch1-IC–infected lineage-negative bone marrow progenitors stained with antibodies to Relb (pseudocolored green) and the nuclear envelope protein Lamb1 (pseudocolored red). Scale bar, 10 μM.

quantitative RT-PCR (qRT-PCR) to characterize gene expression in two cell populations infected with a Notch1-IC–encoding retrovirus: an 'early' population of uncommitted bone marrow progenitors purified 4 d after infection and a 'late' population, prepared by culturing the early-population cells for an additional 7 d on the OP9 stroma line[18] consisting primarily of $CD25^{+}CD44^{low}$ pro-T cells (**Supplementary Fig. 1** online). As a control, we used progenitors infected with an empty, enhanced green fluorescent protein (EGFP)-only retrovirus.

We validated our methodology by examining changes in the expression of known target genes of Notch. Both populations exhibited significantly ($P < 0.001$) increased expression of the Notch targets *Dtx1*, *Nrarp* and *Hey1*. Broad analysis of the gene expression profile was consistent with the role of Notch1 signaling in promoting T-cell fate. The transcription of a number of T lineage–specific genes was markedly increased, even though the early-population cells did not yet exhibit any surface differentiation markers. At the same time, transcription of B cell– and myeloid-specific genes was markedly reduced. Furthermore, the late population showed highly increased expression of additional genes involved in T-cell development and further downregulation of non–T-lineage genes (**Supplementary Fig. 1** and **Supplementary Table 1** online). Together, these results showed that Notch signaling initiates a cascade of transcriptional changes that activate the T-lineage transcriptional program and suppress myeloid and B-cell transcriptional profiles.

Notch1-IC activates NF-κB independently of the pre-TCR

Genes upregulated by Notch1-IC in the early population included several components of the NF-κB pathway, such as *Relb*, *Nfkb2* and *Nfkbia* (*IκBα*), and a number of known target genes of NF-κB. Most of these genes remained upregulated in the late population (**Fig. 1a**). We observed similar transcriptional induction using qRT-PCR assays (**Fig. 1b**). Subsequent immunofluorescence analyses of Notch1-IC–expressing bone marrow progenitors showed an increased amount and enhanced nuclear localization of Relb relative to Notch1-IC–negative bone marrow progenitors (**Fig. 1c**), further confirming the observed transcriptional changes.

NF-κB can be activated by pre-TCR, which is expressed in developing thymocytes and in a minute fraction of the bone marrow progenitor population[19,20]. It has been suggested that the gene encoding the α-chain of the pre-TCR (*Ptcra*) is a transcriptional target of Notch1 signaling[21]; nonetheless, its expression was not induced by Notch1-IC in the early population (**Supplementary Fig. 1**), and fluorescence-activated cell sorting (FACS) analyses showed that several components of the pre-TCR (TCRβ and CD3) were not present on the surface of Notch1-IC–expressing early-population cells (data not shown). To directly address the role of the pre-TCR, we compared the expression of several NF-kB targets in Notch1-IC^{+} progenitors purified from wild-type and $Ptcra^{-/-}$ bone marrow. We did not detect any gene expression differences between the two populations. Furthermore, we found that Notch1-IC^{+} $Ptcra^{-/-}$ bone marrow progenitors, when cultured on OP9 stromal cells, proliferated and differentiated comparably to Notch1-IC–infected wild-type progenitors before the stage of pre-TCR expression and the process of TCRβ selection (**Supplementary Fig. 2** online).

Mechanisms of Notch1-IC-NF-κB pathway interaction

We next sought to determine whether Notch1 could induce NF-κB transcriptional activity. The activation of an NF-κB–responsive luciferase reporter by Notch1-IC was comparable to that by a constitutively active form of Ikbkb ($IKK\beta_{SS\text{-}EE}$) (ref. 22 and **Fig. 2a**). This transcriptional activation was completely blocked by $I\kappa B\alpha^{\Delta N}$, a super-repressor form of Nfkbia. To determine whether activation of the NF-κB pathway requires Notch1-IC–mediated transcription, we used a dominant-negative form of the Notch co-activator MAML1 (DN-MAML1)[23]. Co-transfection of DN-MAML1 along with Notch1-IC resulted in substantially reduced activity of an NF-κB–responsive luciferase reporter (**Fig. 2b**).

These results suggest two possible ways by which Notch1-IC could activate NF-κB: by increasing transcription of the positive regulators of the pathway[24] or, as has been demonstrated for *Notch3* (ref. 7), by directly impinging on the activation of the IKK complex. We tested the first hypothesis using genome-wide chromatin immunoprecipitation assay (ChIP-on-chip) analyses of Notch1 target genes in two T-ALL cell lines (HPB-ALL and CUTLL1; refs. 14,25). We did not detect any significant ($P > 0.1$) binding of Notch1-IC for most of the promoters of the NF-κB targets induced in Notch1-IC–expressing progenitors (**Table 1**). However, the *EGR1*, *NFKB2* and *RELB* promoters presented an exception, because they showed binding ratios that were either significant ($P < 0.03$) (*EGR1* in CUTLL1 line) or could be considered significant ($P < 0.07$) (**Table 1**). Therefore, we further examined Notch1-IC binding to these promoters using conventional ChIP analysis and found that the promoter sequences of *RELB* (in HPB-ALL) and *NFKB2* (in both CUTLL1 and HPB-ALL), but not *EGR1*, were enriched in Notch1-IC immunoprecipitates (**Fig. 2c**). These results suggest that Notch1 can directly bind to *RELB* and *NFKB2* promoters, and are consistent with increased *Relb* and *Nfkb2* expression in Notch1-IC–expressing progenitors (**Fig. 1c**).

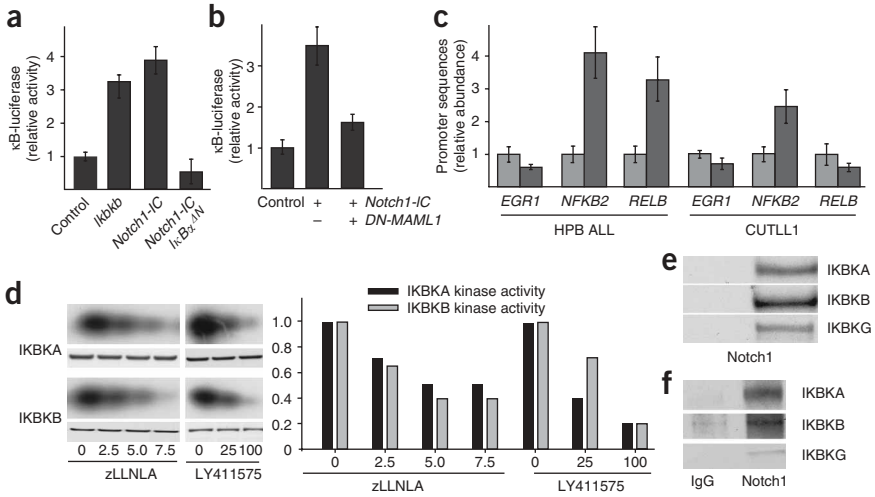

Figure 2 Notch1-IC activates the NF-κB pathway by interacting with the IKK complex and by increasing transcription of *NFKB2* and *RELB*. (**a**) Activity of an NF-κB–responsive luciferase reporter (*3xκB::luciferase*) in COS7 cells transfected with *pCDNA3.1* (control), active *Ikbkb* or *Notch1-IC* with or without $I\kappa B\alpha^{\Delta N}$. (**b**) Activity of an NF-κB-responsive luciferase reporter (*3xκB::-luciferase*) in COS7 transfected with *pCDNA3.1* (control), or *Notch1-IC* with or without *DN-MAML1*. *DN-MAML1* was transfected at tenfold excess relative to *Notch1-IC*. (**c**) Quantitative PCR analyses of whole-cell extracts (input) and chromatin immunoprecipitated with an antibody to Notch1-IC from the human T-ALL lines HPB ALL and CUTLL1. Bars indicate the relative abundance of *EGR1*, *NFKB2* and *RELB* promoter DNA (quantified as a ratio to β-actin genomic DNA). (**d**) Autoradiograms of ^{32}P-labeled NFKBIA after incubation with IKBKA or IKBKB immunoprecipitated from CEM cells treated with the indicated concentrations of the γ-secretase inhibitors Z-Leu-Leu-Nle-CHO and LY411575. Bottom panels show immunoblots of the relative amounts of immunoprecipitated IKKα and IKKβ used for the kinase assays. Right panel shows the relative amounts of phosphorylated IκBα, as determined by densitometry analysis. (**e**,**f**) Western blot detection of Notch1-IC and IKBK(A, B, G) in CEM cell extracts immunoprecipitated with either antibodies to the three IKKs (**e**) or an antibody to Notch1-IC (**f**).

We then addressed the ability of Notch1 to directly interact with the IKK complex. Treatment of human T-ALL–derived cell lines that harbor Notch1-activating mutations[14] with two chemically distinct γ-secretase inhibitors resulted in decreased phosphorylation of a GST-NFKBIA substrate by both IKBKA and IKBKB (**Fig. 2d** and **Supplementary Figs. 3** and **4** online), clearly demonstrating that attenuation of Notch1 signaling affects the activation of the IKK complex in T-ALL cells. Note that the concentrations used of the two γ-secretase inhibitors were able to efficiently silence Notch1 activity and had no effects on cell viability. Activity of the NF-kB pathway, as assessed by the abundance of nuclear, DNA-bound NFKB1 and RELA, was also decreased in response to treatment with the LY411575 γ-secretase inhibitor (**Supplementary Fig. 4**). To further probe the mechanism by which Notch1-IC activates IKK, we performed protein immunoprecipitation assays using antibodies to IKK (α, β and γ) and Notch1-IC. All three subunits of the IKK complex were detected in Notch1-IC immunoprecipitates (**Fig. 2e** and **Supplementary Fig. 5** online), suggesting that Notch1-IC directly interacts with the IKK complex. Reciprocal immunoprecipitation, using IKK antibodies, yielded similar results (**Fig. 2f**).

The NF-κB pathway is activated in human T-ALL

To determine whether activated forms of Notch1 recently identified in human T-ALL were also capable of inducing NF-κB, we created vectors expressing derivatives of human *NOTCH1* containing mutations in the heterodimerization domain (L1601P) and the PEST (P, E, S, T-rich) domain (ΔPEST), separately and in combination (L1601P + ΔPEST), and examined the ability of these mutants to activate CBF1- and NF-κB–responsive reporters. Whereas wild-type *NOTCH1* did not activate a *Cbf1*-controlled reporter, the single mutant forms ΔPEST and L1601P respectively caused a twofold and a ninefold increase in luciferase activity, and the L1601P + ΔPEST form caused a 105-fold increase in reporter activity (**Fig. 3a**), indicating synergism between the two types of *NOTCH1* mutations. Similarly, the mutant forms of *NOTCH1* activated an NF-κB–responsive reporter, with the strongest activation caused by the L1601P + ΔPEST double-mutant. Co-transfection of $I\kappa B\alpha^{\Delta N}$ suppressed NF-κB activation by the mutant forms of *NOTCH1* (**Fig. 3b**).

Table 1 ChIP-on-chip analysis of NOTCH1-IC binding to promoters of NF-κB target genes

				HPB-ALL		CUTLL1	
RefSeq	Gene	Change	Binding	Max. BR	*P*-value	Max. BR	*P*-value
NM_010907	*NFKBIA*	2.1	Not bound	1.4	0.15133	1.15	0.287926
NM_019388	*CD86*	2.4	Not bound	1.79	0.626366	0.87	0.336853
NM_008348	*IL10RA*	2.9	Not bound	1.65	0.231946	1.18	0.648427
NM_008416	*JUNB*	3.3	Not bound	1.15	0.547398	1.44	0.0816662
AK079685	*IRF7*	3.4	Not bound	0.95	0.578127	1.11	0.327223
BC008626	*ICAM1*	4.9	Not bound	1.41	0.388913	1.12	0.600673
AK037968	*CARD11*	7.6	Not bound	1.19	NA	1.09	NA
NM_008091	*GATA3*	8.0	Not bound	1.81	0.102715	1.99	0.032811
NM_009142	*CX3CL1*	10.8	Not bound	1.48	0.209607	1.17	0.148674
NM_008352	*IL12B*	11.0	Not bound	1.21	0.159833	1.14	0.360421
NM_009421	*TRAF1*	15.9	Not bound	1.34	NA	1.09	NA
NM_007719	*CCR7*	27.8	Not bound	1.36	0.179138	0.88	0.785332
NM_013653	*CCL5*	28.0	Not bound	1.59	0.283444	1.16	0.206885
NM_009856	*CD83*	69.2	Not bound	1.14	NA	1.39	NA
NM_007534	*BCL2A1*	94.0	Not bound	1.42	0.433254	1.02	0.637871
NM_019408	*NFKB2*	5.6	Not bound	1.51	0.036291	2.14	0.004589
NM_009046	*RELB*	10.6	Not bound	1.43	0.025912	1.26	0.029515
NM_007913	*EGR1*	5.2	CUTLL1	1.14	0.069905	1.77	0.000826

Change represents fold increase in expression in Notch1-IC$^+$ bone marrow progenitors (see **Fig. 1**, **Supplementary Figs. 1–6** and **Supplementary Table 1**). Binding indicates presence or absence of Notch1-IC binding as determined by combined software-based analysis of absolute binding ratios, relative binding ratios among adjacent probes, and statistical significance (*P*-value). Max. BR represents the maximal binding ratio of Notch1 to the promoters of indicated genes in the T-ALL lines HPB-ALL and CUTLL1. NA, not available.

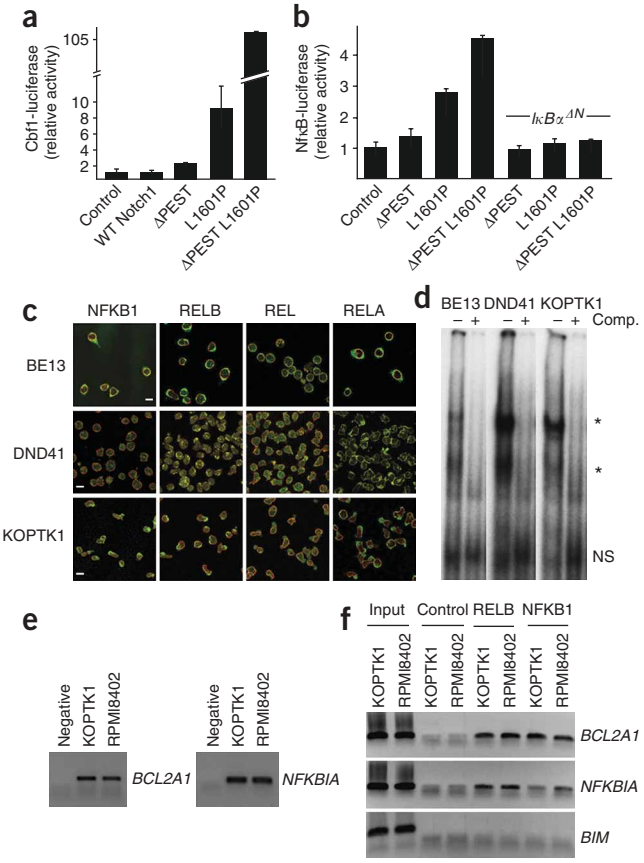

Figure 3 T-ALL–derived mutant forms of Notch1 can activate NF-κB. (**a**) Activity of a *Cbf1*-responsive luciferase reporter in HeLa cells transfected with *pCDNA3.1* (control) or human T-ALL–derived active forms of Notch1. (**b**) Activity of an NF-κB–responsive luciferase reporter (*3xκB::luciferase*) in COS7 cells transfected with *pCDNA3.1* (control) or human T-ALL–derived active forms of *NOTCH1* with or without $I\kappa B\alpha^{\Delta N}$. (**c**) Confocal micrographs of human T-ALL lines stained with antibodies to NFKB1, REL, RELB, RELA (pseudocolored green) and LAMB1 (pseudocolored red). (**d**) EMSA detecting binding of nuclear NF-κB factors on a consensus NF-κB site containing oligonucleotide. Nuclear extracts from the T-ALL lines KOPTK1, BE13, DND41 were used. Asterisks indicate specific nuclear complexes. (**e**) RT-PCR detection of *BCL2A1* and *NFKBIA* expression in the human T-ALL lines KOPTK1 and RPMI8402. (**f**) ChIP assay using antibodies to NFKB1 and RELB, and the T-ALL lines KOPTK1 and RPMI8402. The oligonucleotides were designed to detect NF-kB sites on the promoters of *BCL2A1* and *NFKBIA*. An NF-κB–insensitive promoter (*BIM*) was used as control.

We then used confocal microscopy to examine the subcellular distribution of NF-κB proteins in established human T-ALL lines carrying *NOTCH1* mutations[14]. We observed nuclear localization of NFKB1 (p50), REL (c-Rel) and RELB in all of the lines examined. The RELA factor (p65) appeared to be mostly cytoplasmic, although nuclear staining was also observed (**Fig. 3c** and **Supplementary Fig. 6** online). To directly prove activation of the NF-kB pathway in T-ALL lines harboring *NOTCH1* mutations, we performed both an electrophoretic mobility shift assay (EMSA) and ChIP using unstimulated T-ALL cells. NF-kB complexes were clearly detected in nuclear extracts of DND41, BE13 and KOPTK1 cells (**Fig. 3d–f**). Also, we observed a clear binding of both RELB and p50 on the promoter regions of the *BCL2A1* and *NFKBIA* genes, both targets of the Notch1-NF-kB signaling cascade (**Fig. 1**). These results demonstrate that the NF-kB pathway is constitutively activated in human T-ALL cells that harbor *NOTCH1* mutations.

Human T-ALL lines are susceptible to NF-κB inhibition

These observations prompted us to ask whether these cell lines were susceptible to pharmacological inhibition of NF-κB. The small-molecule compound BMS-345541 potently and specifically inhibits the IKKα kinase both *in vitro* and *in vivo*[26]. Although BMS-345541 had no effect on the transactivation ability of Notch1-IC (data not shown), it efficiently suppressed Notch1-induced activation of the NF-κB pathway (**Fig. 4a**). BMS-345541 caused rapid decline in viability of all four T-ALL cell lines tested (**Fig. 4b**), and this effect was due to increased apoptosis (**Fig. 4c**) rather than cell cycle arrest (data not shown). We observed a similar effect on the viability of T-ALL cell lines with sulfasalazine (data not shown), another NF-κB inhibitor[27].

NF-κB function is also inhibited by broader-acting pharmaceutical agents, such as bortezomib (Velcade), a proteasome inhibitor approved for the treatment of multiple myeloma. The anticancer activity of bortezomib is partly due to the suppression of NF-κB function via the inhibition of proteasome-mediated degradation of Nfkbia[28,29]. We asked whether bortezomib also affects the survival and proliferation of Notch1-dependent human T-ALL lines, and found that five of six tested T-ALL lines were highly sensitive to bortezomib, whereas a severe combined immunodeficient thymocyte cell line (SCIET27), which does not depend on NF-κB activation[19], was resistant to clinically relevant concentrations of bortezomib (**Fig. 4d**). The human leukemia Jurkat line was also resistant to treatment with bortezomib (5 nM) and BMS-345541. The RPMI8402 line seemed to be relatively resistant at this (5 nM) concentration of bortezomib. However, this line was sensitive to higher concentrations of the inhibitor (>25 nM). Bortezomib induced cell death specifically in the T-ALL–derived cell lines (**Fig. 4e**), but did not affect cell cycle progression. To directly prove that bortezomib treatment blocked the NF-kB pathway, we treated T-ALL cells with the inhibitor and performed ChIP using antibodies to NFKB1 and RELB. We found that bortezomib can efficiently suppress the binding of NF-kB to the promoters of the target genes *BCL2A1* and *NFKBIA* (**Fig. 4f**). Together, these results suggested that NF-κB inhibition could be a powerful tool for the treatment of T-ALL.

We then asked whether therapeutic effects could be enhanced by combinatorial inhibition of Notch1 and NF-κB pathways. We treated human T-ALL lines with varying combinations of the γ-secretase inhibitor LY411575 and bortezomib, and found that application of both drugs resulted in a strongly synergistic cytotoxic effect on T-ALL cell lines (CEM, DND41 and KOP-TK1) sensitive to γ-secretase inhibitors[14] or bortezomib (**Fig. 4g**).

NF-κB is important for Notch1-IC–induced T-cell leukemia

To determine whether NF-κB function is required for Notch1-induced transformation *in vivo*, we examined recipient mice transplanted with Notch1-IC–infected bone marrow progenitors derived from either wild-type or *Ikbka*$^{\Delta N}$-transgenic mice (IκBα$^{\Delta N}$). The latter have attenuated NF-κB activity in the T lineage as the result of IκBα$^{\Delta N}$ transgene expression driven by a *p56*^{lck} promoter/*Cd2* enhancer cassette[30]. Induction of leukemia in recipients of wild-type (wt)-Notch1-IC^{+} progenitors resulted in death in as little as 25 d (**Fig. 5a**), with cachexia, crouched posture and reduced mobility becoming apparent about 1 week before death. In contrast in hosts transplanted with IκBα$^{\Delta N}$-Notch1-IC^{+} cells, the disease progressed with significantly ($P < 0.0002$) slower kinetics and reduced severity (**Fig. 5a**). This difference was not due to the lack of progenitor

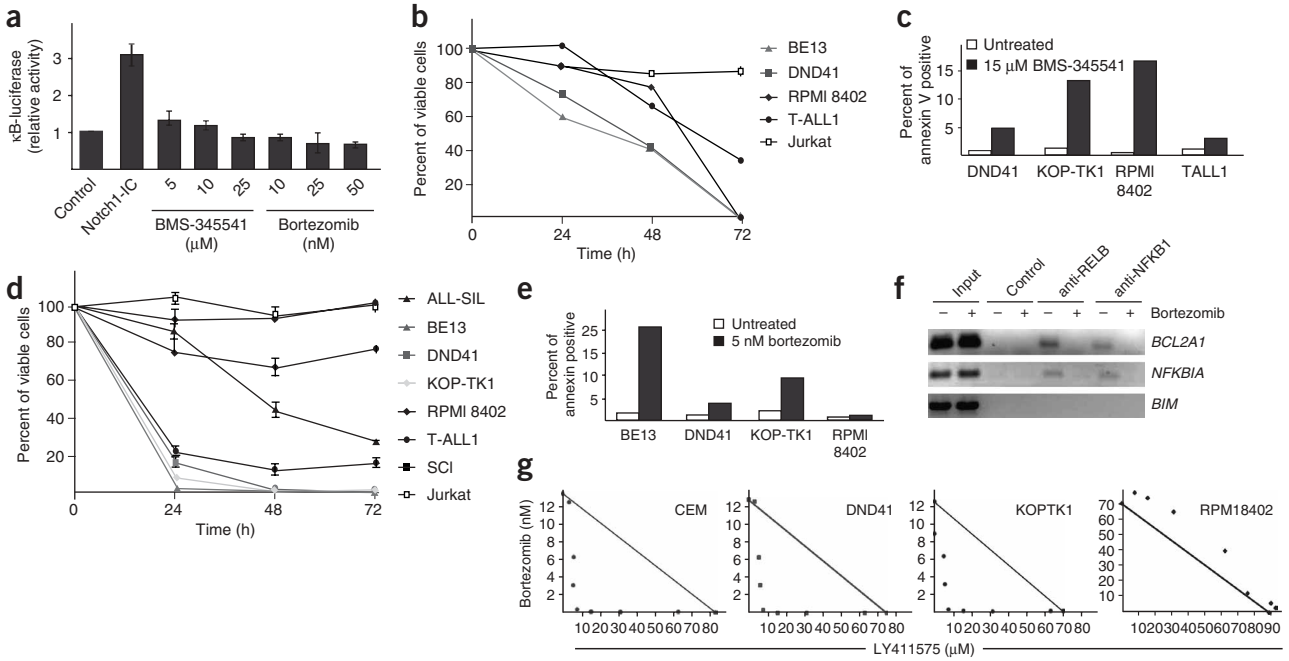

Figure 4 Human T-ALL lines are susceptible to NF-κB inhibition. (**a**) Activity of an NF-κB–responsive luciferase reporter in COS7 cells transfected with *NOTCH1-IC*, and treated for 3 h with the indicated concentrations of BMS-345541 or bortezomib. (**b**,**d**) Cell survival analyses of T-ALL lines cultured in the presence of 15 μM BMS-345541 (**b**) or 5 nM bortezomib (**d**). The percent of viable cells indicates the proportion of live-gated cells in the treated populations relative to untreated populations. (**c**,**e**) Annexin V assay of T-ALL lines treated with BMS-345541 or bortezomib. Bars indicate the proportion of annexin V–positive cells after a 24-h treatment with 15 μM BMS-345541 (**c**) or 5 nM bortezomib (**e**). (**f**) ChIP assay using KOPTK1 T-ALL cells treated for 6 h with 5 nM bortezomib. NFKB1 and RELB antibodies were used. Primers detecting the NF-kB sites on the *BCL2A1* and NFKBIA are used. The *BIM* promoter was used as a control. (**g**) Isobolograms of T-ALL lines treated with both LY411575 and bortezomib. Data points indicate specific LY411575 and bortezomib concentrations at which cell viability declined to 50% after 3–4 d (cytotoxic IC_{50}). Lines connecting cytotoxic IC_{50} for each inhibitor signify a theoretical linear relationship between the inhibitors. Data points below the line indicate additive (synergistic) cytotoxicity; those above the line indicate less-than-additive (antagonistic) cytotoxicity.

engraftment, as $CD4^+CD8^+$ blasts were detectable in the peripheral blood of hosts at 2 weeks after transplantation (**Fig. 5b**). Immunofluorescence demonstrated that whereas wt-Notch1-IC+ blasts showed elevated nuclear p50 relative to nonleukemic $Cd4^+Cd8^+$ thymocytes, the $I\kappa B\alpha^{\Delta N}$-Notch1-$IC^+$ leukemic cells showed a predominantly cytoplasmic localization of p50 (**Fig. 5c**).

To understand how NF-κB deficiency in leukemic T-cell progenitors results in prolonged host survival, we characterized the differences in the distribution and behavior of wt-Notch1-IC^+ and $I\kappa B\alpha^{\Delta N}$-Notch1-$IC^+$ leukemic cells. First, we observed a marked shift in peripheral blood loads of leukemic cells: whereas both wt-Notch1-IC^+ and $I\kappa B\alpha^{\Delta N}$-Notch1-$IC^+$ leukemias had high but comparable levels of $CD4^+CD8^+$ cells in the periphery 2 weeks after transplantation, only the $I\kappa B\alpha^{\Delta N}$-Notch1-$IC^+$ leukemias maintained high levels of $CD4^+CD8^+$ cells in the periphery at 3 weeks after transplantation (**Fig. 5b**). Second, we found that inhibition of NF-κB resulted in reduced tissue infiltration (**Fig. 5d**). Last, we observed that $I\kappa B\alpha^{\Delta N}$-Notch1-$IC^+$ leukemic cells were able to repopulate the thymus of recipient mice, whereas wt-Notch1-IC^+ leukemic cells did not (data not shown). Together, these observations suggest that Notch1-IC–induced NF-κB activity leads to changes in migratory response, thereby increasing the infiltrating capacity of Notch1-IC–transformed T-cell progenitors.

NF-κB activation is not sufficient for T-cell leukemia

To determine whether increased NF-κB signaling is sufficient to cause T-cell leukemia in the absence of activated forms of Notch1, we followed leukemia development in $Rag2^{-/-}$ $Il2rg^{-/-}$ hosts transplanted with wild-type bone marrow progenitors carrying a gain-of-function *Ikbkb* transgene ($Ikbkb_{SS\text{-}EE}$)[22], which acts in a similar manner to Notch1-IC in that it induces comparable expression of a NF-κB–responsive luciferase reporter (**Fig. 2a**). Although we were able to confirm that efficient hematopoietic reconstitution had taken place, we did not observe any signs of leukemia over the course of 32 weeks: the peripheral blood of host mice was free of $CD4^+CD8^+$ leukemic blasts (**Fig. 5e**), and all hosts survived to the end of the observation period without adverse changes in their overall health status. Therefore, we concluded that although the NF-κB pathway is important for the establishment of the disease, it is not sufficient to give rise to T-cell leukemia in the absence of activating Notch1 mutations.

DISCUSSION

Our experiments identify the NF-κB signaling pathway as a new target of Notch1 and demonstrate that the ability of Notch1 to activate NF-κB is independent of antigen receptor signaling (pre-TCR). We have proposed two mechanisms of NF-kB induction by Notch1: the direct transcriptional activation of *Relb* and *Nfkb2*, and the biochemical interaction of Notch1 with the IKK complex. Furthermore, we have established that mutated forms of Notch1 found in T-ALL individuals can activate the NF-κB pathway and have demonstrated that the NF-κB pathway can be a promising molecular target for the treatment of T-ALL.

However, our experiments demonstrate that NF-κB is only a part of the Notch puzzle. Indeed, expression of an activated form of the IKK

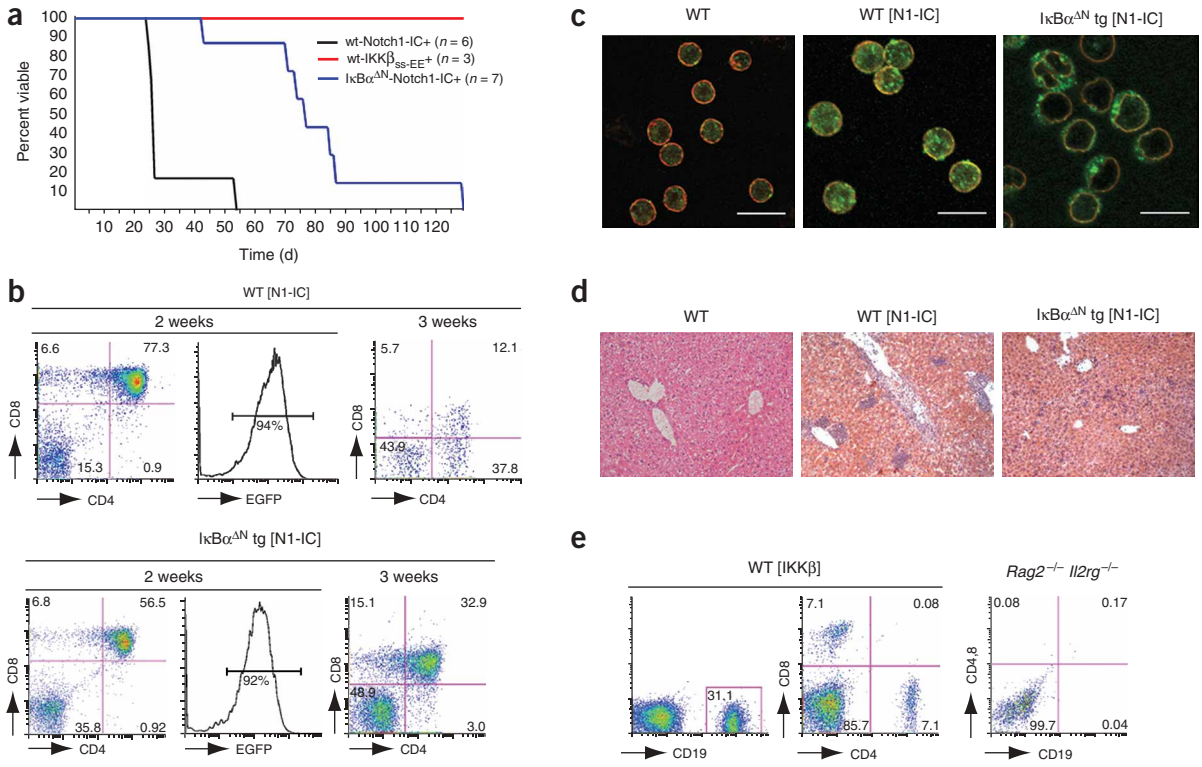

Figure 5 NF-κB is important but not sufficient for T-ALL development *in vivo*. (**a**) Kaplan-Meyer survival curve of mice transplanted with wt-Notch1-IC$^+$, IκBα$^{\Delta N}$ Notch1-IC$^+$ or IKKβ$^*_{SS\text{-}EE}$ bone marrow progenitors. (**b**) Flow cytometric analyses of CD4$^+$CD8$^+$ Notch1-IC$^+$ leukemic cells in the peripheral blood of host mice at indicated times after transplantation. Histograms indicate the proportion of CD4$^+$CD8$^+$ cells (left panels, upper right quadrants) carrying EGFP-marked Notch1-IC. (**c**) Confocal micrographs of wild-type nonleukemic CD4$^+$CD8$^+$ thymocytes and Notch1-IC$^+$ CD4$^+$ CD8$^+$ leukemic cells purified by FACS from the spleens of host mice and stained with antibodies to NFKB1 (pseudocolored green) and Lamb1 (pseudocolored red). Scale bar, 10 μM. (**d**) Micrographs of hematoxylin/eosin-stained liver sections prepared 19 d after transplantation of wt-Notch1-IC$^+$ or IκBα$^{\Delta N}$-Notch1-IC$^+$ bone marrow progenitors. (**e**) Flow cytometric analyses of reconstitution of lymphoid lineages in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice transplanted with IKKβ$^*_{SS\text{-}EE}$ bone marrow progenitors.

kinase did not induce T-cell leukemia, and *in vivo* attenuation of NF-kB activation did not provide complete protection from leukemia. These experiments suggest that Notch activates additional molecular pathways that can synergize with NF-kB to induce oncogenic transformation. One likely candidate could be *Myc* (*c-Myc*), which is transcriptionally regulated by Notch1 in both breast cancer and T-cell leukemia[31,32]. Another potential cooperating pathway could be the Akt/PKB cascade that is activated by Notch1-IC (I.A. and M.M., unpublished data). Akt activation could either further activate the NF-kB pathway or regulate several downstream targets (such as *Foxo3a*, *Cdkn1b*, *Gsk3b* and *Ccnd1*) that could facilitate transformation.

How does Notch1 regulate NF-κB function? Previous reports have proposed direct interactions of Notch1-IC with NF-κB subunits[33–35], and very recent work has demonstrated that Notch1-IC interacts with NF-κB and competes with the IkBα protein, enhancing the retention of Nfkb1 and Rel in the nucleus[36]. Our observations suggest that Notch1 also regulates the NF-κB pathway by inducing the expression of *Relb* and *Nfkb2*, and by directly stimulating the activity of the IKK complex. In addition, activation of NF-κB may also be mediated by *Notch3*. Recent work shows that Notch3 can activate the NF-κB pathway by phosphorylating IKKα homodimers, which in turn activate the noncanonical p52-Relb NF-κB pathway[7]. Moreover, our findings and previous results[25] provide evidence that *Notch3* is a direct transcriptional target of Notch1. Thus, in principle, Notch-1 could activate the NF-κB canonical signaling by activating the IKKα/β/γ signalosome and facilitating the nuclear retention of NF-κB heterodimers, and the noncanonical pathway by inducing the expression of Relb and Nfkb2 and activating IKKα homodimers via Notch-3.

The discovery of activating Notch1 mutations in T-ALL has made the Notch pathway an attractive target for therapy; indeed, small-molecule inhibitors of the γ-secretase complex have already shown potential for the treatment of Notch-induced malignancies and are currently in clinical trials[37,38]. The results described here indicate that inhibition of the NF-κB pathway also represents a potentially useful strategy for T-ALL treatment. Indeed, as our study has shown, bortezomib as well as other NF-κB–inhibiting agents are attractive candidates for T-ALL treatment, either as single drugs or in combination with γ-secretase inhibitors. The strongly synergistic effect of bortezomib with the latter agents suggests the attractive possibility of a combination regimen in which each agent is used at reduced dosages.

METHODS

Mice and animal procedures. C57BL/6 and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice were purchased from Jackson Laboratories and Taconic Farms, respectively. *IκBα*$^{\Delta N}$ and *Ptcra*$^{-/-}$ mice have been described previously[30,39]. All mice were kept in specific pathogen-free animal facilities at the University of Chicago. All animal procedures were performed in accordance to the guidelines of the Institutional Animal Care and Use Committee of the University of Chicago.

Antibodies and reagents. All primary and secondary FACS antibodies were purchased from BD Biosciences. We also used antibodies to Nfkb1 (Abcam), Ikbka (Imgenex), Ikbka/g, Rela, Rel, Relb and Lamb1 (Santa Cruz

Biotechnologies). Antibodies to the intracellular fragment (active form) of Notch1 were purchased from Abcam and Cell Signaling. GST-IκBα was purchased from Upstate. Cytokines were obtained from Peprotech. LY294002, BMS-345541 and the γ-secretase inhibitor Z-Leu-Leu-Nle-CHO were purchased from EMD Biosciences. Velcade was provided by the pharmacy of the University of Chicago Hospitals.

Recombinant DNA constructs and retrovirus production. IκBα$^{\Delta N}$ retroviral vector was created by cloning a PCR-amplified *Nfkbia* sequence encoding amino acids 37–317 into a CMMP IRES-EGFP vector. Expression vectors carrying haemagglutinin (HA)-tagged *Notch1* L1601P and ΔPEST mutants were created by multistep insertion of PCR-amplified human *NOTCH1* sequences into pcDNA3.1 (Invitrogen). The Notch1-IC retroviral plasmid[40], its parent vector CMMP, and the *Ikbka* (IKKβ$_{SS\text{-}EE}$) retroviral plasmid (described in ref. 22) were used for infection of bone marrow progenitors and cell lines. The DN-MAML1 vector has been described previously[14]. Viral supernatants were generated as described previously[41].

Isolation and retroviral infection of bone marrow lineage-negative progenitors. Isolation, retroviral infection and reconstitution experiments were performed as previously described[42].

OP9-DL1 coculture. Culture of bone marrow progenitors on OP9 cells was performed as previously described[43].

Microarray and quantitative RT-PCR. Accession numbers for individual array comparisons are GSE6396 – GSM147443, GSM147464 and GSM147508. Total cellular RNA was isolated using RNAqueous Micro (Ambion), or RNeasy (Qiagen) columns. RNA quality analyses, labeling, microarray hybridization, fluorescence normalization, dye-swaps and gene expression, and *P*-value calculations were carried out using Icoria (Clinical Data). Gene expression was analyzed using the 7300 Real-Time PCR System and Sequence Detection Software version 1.2.2 (Applied Biosystems). Relative expression was determined from cycle threshold (C_T) values and was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous reference. The following primers were used for quantitative RT-PCR experiments: mouse *Bcl2a1* forward, 5′-CATTAACTGGGGAAGGATTGTGAC-3′, and reverse, 5′-GCAGAAAAGTCAGCCAGCCAGATT-3′; *Ddr1* (*Cak*) forward, 5′-GGCCATC ATCCTGCTGCTGCTTCT-3′, and reverse, 5′-GGGGTCCTGGGCGGTTGTT GAT-3′; *Ccr7* forward, 5′-CTACAGCCCCCAGAGCACCAT-3′, and reverse, 5′-CCAGGCCTTAAAGTTCCGCACATC-3′; *Egr1* forward, 5′- TGAGAAGGCGAT GGTGGAGACGAG -3′, and reverse, 5′- TGGAGAAGGCGCCGAGGATGAA GA -3′; *Egr2* forward, 5′-CACAGCCTCCTCCAGCGTCACC-3′, and reverse, 5′-AGATCTCCTGTACAGCCCGAATAA 3′; *Gapdh* forward, 5′-ATCACTGCCACC CAGAAGAC-3′, and reverse, 5′-CACATTGGGGGTAGGAACAC-3′; *Gata3* forward, 5′-GGTCGGCCAGGCAAGATGAGAAA-3′, and reverse, 5′-AGGCT GCTGGGTGGGAAGAGTC-3′; *Gfi1* forward, 5′- AGACCCTTTGCGTGCGAG ATG-3′, and reverse, 5′- TGCCACAGTACTGACAGGGATAGG-3′; *Hey1* forward, 5′-AGAAGCGCCGACGAGACCGAATCA 3′, and reverse, 5′-CAG GGCGTGCGCGTCAAAATAACC-3′; *Icam1* forward, 5′-CCGTGGGGAGGAG ATACTGA-3′, and reverse, 5′-CCTGGCCTCGGAGACATTAG-3′; *Id2* forward, 5′-CAAAACCCCGGTGGACGAC-3′, and reverse, 5′-TGGTGATGCAGGCTG ACGATAGT-3′; *P2ry10* forward, 5′-CAATTGCAATGCCACTTAT-3′, and reverse, 5′-CTGCCACTGAGAGGTTGAT-3′; *Rras2* forward, 5′-GGCGAGGGCT TCCTGTTGGT-3′, and reverse, 5′-ACCTTGAGCTGCCTTGCTAACTGC-3′; *Nfkbia* forward 5′-CGGCCTGGA CTCCATGAAAG-3′; *Nfkbia* reverse 5′-CCTT CAC CTGGCGGATCACT-3′;

Immunofluorescence (IF) analysis. Immunostainings were performed as previously described[43]. Images were acquired on a Leica SP2 AOBS confocal station and analyzed using ImageJ software.

Luciferase assays. Luciferase reporter assays were performed as previously described[14]. Luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega) and a 2020n luminometer (Turner Biosystems) configured for dual assays.

Chromatin immunoprecipitation (ChIP). ChIP was performed as described previously[44]. ChIP-on-chip was performed following Agilent Technologies' standard protocols using the Agilent Human Proximal Promoter Microarray platform. The arrays were scanned using an Agilent scanner, data were extracted using the Feature Extraction 8 software and analyzed using ChIP Analytics 1.1.

Enrichment of candidate target promoters was assessed by quantitative PCR analysis of promoters of interest in Notch1-IC chromatin immunoprecipitates and their corresponding whole-cell extracts (input control) using an unrelated β-actin genomic sequence as reference. The following primers were used for detection of Notch1-bound promoter sequences: *Nfkb2* forward, 5′-CTTCAGA GAAAGCCAAGCGTTAG-3′, and reverse, 5′-CTGAGAGGGGACAGGCTC ACT-3′; *Egr1* forward, 5′-CTCTGGGTCT GGGCTTCC-3′, and reverse, 5′-CT CCCTCCTCCCTGGTTC-3′; *Relb* forward, 5′-GGCGTGATGGCTCTAAGCA-3′, and reverse, 5′-CTGGGGAACGGGAAAACC-3′.

For Nfkb1 and Relb ChIP, we used the following primers: *Bcl2a1* forward (ChIP) 5′-TGGAGACAG AGTCTCGCTCTGTT-3′; *Bcl2a1* reverse (ChIP) 5′-GAACCCGGGA GACAGAAGTTG-3′; *Nfkbia* forward (ChIP) 5′-GGCTCAT CGCAGGGAGTTT-3′; *Nfkbia* reverse (ChIP) 5′-GAACTGGCTTCGTCCTCTG CTA-3′; *Bim* forward (ChIP) 5′-GCCTTCTGAGGCTTCCAACT-3′; *Bim* reverse (ChIP) 5′-GCCTG CTCTTGAGA CTCTGC-3′.

Kinase assays and immunoprecipitation. Immunoprecipitation and kinase assays were carried out as described previously[45].

Cytotoxicity assays. Dual-inhibitor cytotoxicity was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 200 μl of a 2×10^5 cell/ml suspension was seeded in 96-well plates in four replicates and incubated for 3–4 d in the presence of either LY411575 (7.82 μM, 15.63 μM, 31.25 μM or 62.5 μM), bortezomib (3.13 nM, 6.25 nM or 12.5 nM) or both. On the day of the assay, 20 μl of a 4mg/ml MTT solution in PBS was added to each well, and the plates were incubated for an additional 3–4 h. Formazan crystals were collected by centrifugation and dissolved using 200 μl of 100% DMSO, and concentration readings (optical density at 540 nm) were obtained using an ELISA plate reader. Cytotoxic half maximal inhibitory concentration (IC_{50}) values were obtained using TableCurve software, by plotting growth curves for cells incubated in the presence of either or both inhibitors.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

I.A. supervised the project. T.V., J.M., T.P., M.M., S.B., F.M., B.T., C.S. and S.M. conducted experiments. M.-L.A. helped with the Ikbia experiments. B.L.K. supervised the EMSA experiments. A.F. supervised the CHIP-on-chip experiments. L.M. supervised experiments and helped with the editing of the manuscript. I.A. and T.V. cowrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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