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The Notch/Hes1 pathway sustains NF- κ B activation through *CYLD* repression in T cell leukemia

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SUMMARY

It was previously shown that the NF- κ B pathway is downstream of oncogenic Notch1 in T cell acute lymphoblastic leukemia (T-ALL). Here we visualize Notch-induced NF- κ B activation using both human T-ALL cell lines and animal models. We demonstrate that *Hes1*, a canonical Notch target

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and transcriptional repressor, is responsible for sustaining IKK activation in T-ALL. *Hes1* exerts its effects by repressing the deubiquitinase *CYLD*, a negative IKK complex regulator. *CYLD* expression was found to be significantly suppressed in primary T-ALL. Finally, we demonstrate that IKK inhibition is a promising option for the targeted therapy of T-ALL as specific suppression of IKK expression and function affected both the survival of human T-ALL cells and the maintenance of the disease *in vivo*.

INTRODUCTION

NF- κ B is an important regulator of cell survival, proliferation and differentiation and is frequently involved in malignant transformation (Karin, 2006). NF- κ B is mainly activated in response to pro-inflammatory stimuli such as TNF α or IL1 β , components of pathogenic bacteria and viruses or following TCR signaling in T-lymphocytes. Regardless of the activation of specific receptors that associate to different effectors, canonical NF- κ B signaling invariably leads to the activation of I κ B kinase (IKK) complex composed by two catalytic and one regulatory subunits called IKK α , IKK β and NEMO (IKK γ), respectively. The main target of activated IKK complex is the NF- κ B inhibitor, I κ B, that it is rapidly phosphorylated in specific serine residues thus targeting the protein for ubiquitination, leading to proteasomal degradation. Degradation of I κ B is sufficient to induce the nuclear translocation of the transcription factor that results in specific gene activation (Vallabhapurapu and Karin, 2009)). I κ B α itself is one of the main transcriptional targets for NF- κ B activity thus providing an efficient mechanism for signal termination (Hoffmann et al., 2002). However, other mechanisms are required to maintain the non-active state of NF- κ B in the absence of stimulation but also to attenuate the signal following chronic stimulation of the pathway. They involve the deubiquitination enzymes A20 and CYLD, which remove the activator K63 ubiquitin chains from different elements of the NF- κ B signalosome (Brummelkamp et al., 2003; Reiley et al., 2007; Trompouki et al., 2003; Wertz et al., 2004).

Despite the huge amount of gathered data on NF- κ B activation, not much is known about the crosstalk of NF- κ B with other signaling pathways, including pathways that control tissue differentiation like the Notch cascade. Notch receptors are crucial regulators of cell differentiation in multiple systems (Reviewed by (Egan et al., 1998)). Activation of Notch by Jagged or Delta ligands results in the cleavage of the intracellular fragment of the receptor by a γ -secretase activity (Mumm et al., 2000). Once released from the extracellular part of the molecule, the intracellular cleaved Notch translocates to the nucleus to bind the ubiquitous RBPj κ factor and to activate transcription of a number of target genes, including the *Hes* family of transcription factors (Iso et al., 2003; Jarriault et al., 1995). The classical function of *Hes* family proteins is the transcriptional repression of pro-differentiation genes by direct interaction with specific sequences of their promoters or by the association with other bHLH factors and combinatorial DNA binding (Kageyama and Ohtsuka, 1999; Kuroda et al., 1999). The Notch pathway was recently shown to be oncogenic in a wide variety of cancers, most notably in T cell acute lymphoblastic leukemia (T-ALL), a devastating pediatric tumor arising by the transformation of hematopoietic progenitor cells. In this disease, the majority of patients harbor either activating mutations of Notch1 or inactivating mutations affecting negative regulators of the Notch pathway (Aifantis et al., 2008)

Recent evidence has suggested an interaction between Notch and NF- κ B, more specifically in the context of cell transformation. Cross-talk between the Notch and NF- κ B pathways have been proposed in human T-ALL and in mouse models of T cell leukemia (Screpanti et al., 2003; Shin et al., 2006; Vilimas et al., 2007). More specifically, we have previously shown that Notch activation can induce the expression of a large fraction of classical NF- κ B gene targets in T cell progenitors. We have also demonstrated that this Notch-induced NF- κ B

activation requires both the transcriptional activity of Notch and the function of the IKK complex (Vilimas et al., 2007). However, these studies failed to provide a definitive demonstration that this interaction can be translated into a new therapeutic tool. Moreover, neither the mechanism explaining the ability of Notch to impinge on IKK (and thus NF- κ B) activation nor the importance of IKK activation for the maintenance of T-ALL *in vivo* was addressed. These key issues could introduce NF- κ B inhibition as an effective therapy for the treatment of T-ALL and are all addressed here.

RESULTS

Oncogenic Notch induces NF- κ B activation both *in vitro* and *in vivo*

We attempted here to visualize *in vivo* the Notch-induced NF- κ B activation using an animal model of T-ALL. We combined an established transplantation model with a NF- κ B-dependent *in vivo* luciferase reporter strain (κ BLuc) to measure the activation of this pathway. We transplanted ICN1⁺ κ BLuc⁺ progenitors in irradiated hosts and monitored the progression of the disease and the activation of the reporter using both FACS analysis and *in vivo* bioluminescence. Disease was induced as evidenced by the appearance of leukemic CD4⁺CD8⁺ blast cells in the peripheral blood (not shown). Early during T-ALL, visual induction of NF- κ B activation was undetectable, probably due to the low number of transformed cells at this stage of the disease. However, NF- κ B activity increased rapidly and was readily detectable 3 wks post-cell transplantation with “hotspots” in tissues containing lymphoid cells, including the thymus and bone marrow areas (Figure 1A).

To further prove that Notch was responsible for induction of NF- κ B activation, we used a distinct, recently generated animal model of the disease that does not require transplantation. In this model, the oncogenic ICN1 mutant is “knocked-in” the *EF1a* locus and induced by cre-recombinase expression (Buonamici et al., 2009). ICN1 expression rapidly induced T-ALL (Figure 1B) that overexpressed nuclear Notch1-IC and its target gene *Hes1* (Figures 1C, D). To test whether NF- κ B was activated in the leukemic cells, we sorted the CD4⁺CD8⁺ population and used a conventional EMSA approach. As shown in Figure 1E, this leukemic population showed significant NF- κ B activation, as indicated by the increased DNA-binding activity. Antibody shift experiments demonstrated a predominance of p50-p50 (Nfkb1:Nfkb1) and p50:p65 (Nfkb1:RelA) dimers.

To support that Notch is responsible for promoting NF- κ B activity in T-ALL cells, we measured the activation of a virally-driven NF- κ B-dependent EGFP reporter in a representative human cell line (KOPT-K) that carries Notch mutations (O’Neil et al., 2007). We either kept the cells untreated or treated them with gamma-secretase inhibitors (GSI). Inhibition of the Notch pathway by GSI reduced the activation of the NF- κ B-dependent EGFP reporter, the expression of the Notch-target genes *Hes1*, *Deltex1*, the NF- κ B target *Bcl2A1*, as well as NF- κ B DNA binding activity (Figure S1A–C).

These combined experiments support the hypothesis that NF- κ B is targeted by Notch and demonstrate that this cross-talk between the two pathways takes place also in *in vivo* models of T-ALL. We were thus curious to see whether this *in vivo* Notch-induced NF- κ B activation leads to the activation of a subset of genes that have been characterized as direct or indirect NF- κ B targets. We have thus performed a meta-analysis of the transcriptional profiling comparisons between “control” and “leukemic” T cells performed by Li et al. (Li et al., 2008). As shown in Figure 1F, a large number of known NF- κ B target genes, as proposed by multiple studies, were significantly induced in the CD4⁺8⁺Notch1-IC⁺ tumor samples. To further identify genes induced in leukemia that are direct NF- κ B targets, we have combined our analysis to a recent report that combined whole-genome chromatin immunoprecipitation data (ChIP-seq) and RNA-sequencing data, identifying NF- κ B p65 direct transcriptional

targets (Kasowski et al., 2010). As shown in Figure S1D, we identified a significant number of NF- κ B direct targets that are significantly upregulated in T-ALL cells. The combination of these data strongly suggest that a NF- κ B gene expression “signature” is characterizing leukemic T cells in Notch-induced T-ALL.

Hes1 induces NF- κ B activity and nuclear translocation of p65 by facilitating I κ B α degradation

Next, we addressed the mechanism of Notch-induced NF- κ B activation. To answer this question, we measured the activity of a NF- κ B-dependent reporter in response to ectopic expression of ICN1 or its main target *Hes1*. We found that ectopic expression of doxycycline-inducible *Hes1* (Figure 2A) or non-inducible flag-*Hes1* resulted in a dose-dependent activation of NF- κ B, comparable to that induced by ICN1 (Figure S2). Activation of NF- κ B was concomitant with the nuclear accumulation of p65 in *Hes1* expressing cells, as detected by immunofluorescence (Figure 2B).

We analyzed whether modification of the I κ B α protein levels were responsible for the increased NF- κ B-transcriptional activity induced by *Hes1*. We found that ectopic *Hes1* expression correlated with a reduction in I κ B α protein levels in the absence of stimulation that was further enhanced by chronic TNF α treatment (Figure 2C). Most importantly, IF staining of HEK-293T cells demonstrated that after TNF α treatment there was a complete absence of endogenous I κ B α in cells expressing ectopic *Hes1*, whereas the rest of the cells have already re-synthesized I κ B α (Figure 2D).

As *Hes1* is a well-characterized transcriptional repressor (Iso et al., 2003), we speculated that *Hes1* was regulating I κ B α at the level of transcription. To test this possibility, we performed qRT-PCR from cells transfected with the control vector or the inducible *Hes1* construct, untreated or treated with TNF α and/or doxycycline as indicated. We found that *Hes1* expression did not exert any inhibitory effect on either basal or TNF α -induced I κ B α transcription, but rather increased I κ B α transcription as it is an NF- κ B target (Figure 2E). These results suggested that *Hes1* was modulating I κ B α protein stability. In agreement with this, incubation with the proteasomal inhibitor MG132 or mutation of lysines 21 and 22 of I κ B α into arginine, which are required for K48-linked ubiquitination (Rodriguez et al., 1996), completely abrogated the effects of *Hes1* on I κ B α levels (Figure 2F and 2G). These results demonstrated that *Hes1* is able to induce I κ B α degradation, which results in increased NF- κ B activity.

Hes1 facilitates I κ B α degradation by modifying IKK activity

As it is well established that I κ B α degradation is regulated by the IKK complex, in response to the specific phosphorylation by IKK β , we tested the effect of the IKK β inhibitor BAY65-5811 on *Hes1*-induced degradation of I κ B α . Inhibition of the IKK activity completely blocked the phosphorylation of IKK and the degradation of I κ B α induced by ectopic expression of *Hes1* in the presence of chronic TNF α treatment (as detected with the specific antibody recognizing phosphorylated serines 180 and 181 of IKK α and IKK β respectively and I κ B α) (Figure 3A). Next, we tested whether *hes1* expression affected the phosphorylation/activation status of IKK α or β that are the catalytic subunits of the IKK complex. Figure 3B shows that doxycycline-inducible *Hes1* expression significantly and specifically increased the amounts of active/phosphorylated HA-IKK β but not HA-IKK α . As a control we show that this effect was not induced by doxycycline alone (Figure S3A). To further demonstrate the requirement of IKK β in the *Hes1*-induced degradation of I κ B α , we expressed HA-I κ B α together with the inducible *Hes1* construct in IKK β -deficient MEFs. As shown in Figure 3C, *Hes1* was not sufficient to induce I κ B α degradation in cells lacking IKK β , whereas reintroduction of IKK β restored the ability of *Hes1* to reduce I κ B α levels. Finally, ectopic expression of the dominant negative form of TAK1 (TAK1K63W), the specific kinase that activates IKK β , was sufficient to block the I κ B α -degradation imposed by *Hes1* (Figure 3D). These results indicate that *Hes1*

induces I κ B α degradation by facilitating the activation of the IKK complex in both basal conditions and following TNF α treatment, and that this effect takes place upstream of IKK β phosphorylation by TAK1.

Hes1 is responsible for sustaining IKK/NF- κ B activity in Notch-dependent leukemia

Based on these results, we speculated that the NF- κ B activation that occurs in T-ALL with aberrant Notch activity might be mediated through Hes1. We confirmed that Notch1 activation (as detected by the Val1744 antibody) was mainly, although not exclusively, found in T-ALL lines, inversely correlating with the protein levels of I κ B α (Figure S3B). To determine the contribution of Hes1 to the regulation of I κ B α in T-ALL, we infected Jurkat cells with a lentiviral construct containing the shRNA against *Hes1* or the control shRNA. *Hes1* knockdown resulted in increased I κ B α levels in non-induced cells and even more significant after chronic TNF α treatment (Figure 4A). Comparable results were obtained by inhibiting Notch activation in Jurkat cells with GSI (Figure 4B). Moreover, chronic activation of the endogenous IKK kinase complex following TNF α treatment was eliminated in cells with reduced *Hes1* expression (Figure 4C). Next, we tested whether NF- κ B activation was regulated by Hes1 in these cells and found a reduction in the levels of several NF- κ B-target genes such as *Bcl2A1* or *cIAP2* by knocking down *Hes1* in both Jurkat and most important, in primary human T-ALL cells (Figure 4D and 4E). Identical effects were obtained in Jurkat cells treated with GSI (Figure S4A). To study the functional relevance of Hes1 in cells containing active Notch *ex vivo*, we transduced lineage-depleted bone-marrow cells with a lentiviral vector expressing the oncogenic ICN1 alone or together with the *Hes1*-shRNA construct. Cells were serially plated every week and counted as an estimated measure of self-renewal capacity (Lavau et al., 1997) (Figure 4F). We found that ICN1-expressing cells exhibited a higher clonogenic capacity than the cells co-expressing the *Hes1*-shRNA as indicated by the increased number of colonies in these cultures (Figure 4G). Importantly, ICN1-transduced progenitors were able to produce increasing number of CD3⁺ cells when plated on OP9-Dll1 stroma compared to *Hes1*-shRNA-expressing cells (Figure 4H and Table S1). Moreover, in the absence of Hes1, cells expressing ICN1 showed an elevated number of apoptotic cells as measured by Annexin-V binding (Figure S4B) concomitant with a decrease in the expression of the pro-survival genes *Bcl2A1* and *cIAP2* (Figure 4I). To determine whether NF- κ B provided pro-survival signals in ICN1-transduced cells, we plated these cells in the presence of the IKK inhibitor BAY65-5811. In these conditions, expression of oncogenic ICN1 was not sufficient to support colony and cell growth (Figure 4J).

The expression of the deubiquitinase CYLD is suppressed in human T-ALL

Since our results indicated that Hes1 was able to maintain IKK complex activation in T-ALL and we mapped this function upstream of TAK1, we tested whether the expression of the negative regulators of IKK activity, CYLD and A20, was decreased in human T-ALL. In this screen we included a group of 13 healthy control samples (9 whole peripheral blood (WPB) and 4 purified CD3⁺) and 33 WPB samples from T-ALL patients containing more than 60% of leukemic blasts, except sample number 18 with 44% leukemic cells (for details see Table S2). Using qRT-PCR we found that mRNA levels of both deubiquitinating enzymes were significantly decreased in the leukemic samples compared to the normal controls ($p=0.001$ for *CYLD* and $p<0.001$ for *A20* using Student t-test) concomitant with a significant increase in the expression of *Hes1* ($p=0.001$) (Figure 5A). Further meta-analysis of ALL gene expression databases showed that *CYLD* levels in T-ALL and significantly lower than in any B-ALL category (not shown). Next, we tested whether there is an inverse correlation between Hes1 and either *CYLD* or *A20* genes in T-ALL by using *Hes1*-specific shRNA-mediated knock-down. Reduction of *Hes1* levels resulted in a pronounced increase in the mRNA of *CYLD* whereas *A20* levels remained unaffected (Figure 5B). In contrast, overexpression of *Hes1* in HEK-293T cells resulted in a reduction in the levels of *CYLD* mRNA (Figure 5C) and protein

(Figure 5D) associated with lower levels of $\text{I}\kappa\text{B}\alpha$, being this effect more dramatic after chronic treatment with $\text{TNF}\alpha$ (Figure 5D). We next tested whether *Hes1* protein levels inversely correlated with *CYLD* protein levels in different T-ALL cell lines containing Notch1 mutations. As shown in Figure 5E, *CYLD* levels were reduced in T-ALL cells when compared to B-cell (Nalm6, RAJI and RPMI1783) or myeloid leukemia cell line (HL60) concomitant with variable, but higher levels of *Hes1* protein. Importantly, inhibition of Notch1 by GSI resulted in an increase of both protein (Figure S5) and mRNA (not shown) levels of *CYLD* in these cells.

The Notch pathway directly controls *CYLD* expression through *Hes1*

These data suggested that *CYLD* expression is kept suppressed in T-ALL, leading to an enhancement of IKK activity. To test whether the Notch pathway and specifically *Hes1* was directly repressing *CYLD* transcription, we used the Genomatix software to identify putative *Hes1* binding sites, and found three distinct N-box consensus sites in the promoter and 5'UTR of the *CYLD* transcriptional start site (Figure 6A, denoted as PRO1 and PRO2). Using a conventional ChIP assay in human *Hes1*⁺ T-ALL cells and a *Hes1* antibody followed by PCR with specific primers flanking the identified putative N-box sites, we were able to show that endogenous *Hes1* binds to the predicted PRO2 site in the *CYLD* 5'UTR (Figure 6B) and this association was lost following *Hes1* knock-down (Figure 6C) as measured by qPCR. Ectopic expression of *CYLD* completely reverted the clonogenic growth of lineage negative BM cells transduced with ICN1 whereas downregulation of *CYLD* was sufficient to increase the number of colonies in this assay (Figure 6D). Consistent with these results, we found that ectopic expression of *CYLD* is sufficient to block Notch-induced activation of an NF κ B-dependent reporter in HEK-293T (Figure S6A). Moreover, the capacity to produce CD3⁺ T cells by ICN1-transduced progenitors in co-culture on OP9-Dll1 stroma is partially mimicked by *Cyld*-shRNA-expressing cells and lost in cells co-expressing ICN1 and *CYLD* (Figure 6E and Figure S6). To further support the notion that *CYLD* expression in T-ALL is controlled by direct transcriptional repression by *Hes1*, we have quantified CpG methylation at the *CYLD* using the previously introduced human T-ALL samples. As shown in Figure 6F, there was no evidence of promoter hypermethylation suggesting that it is unlikely that DNA methylation can explain by itself the suppression of *CYLD* expression. These observations suggest that suppression of *CYLD* enhances the activity of the IKK kinase and the nuclear localization of NF- κ B transcription factors. To directly test this hypothesis, we have totally eliminated *CYLD* expression in T-ALL using an animal model of *CYLD* deficiency (Reiley et al., 2006). As shown in Figure S6B–D, *CYLD* deletion is sufficient to significantly enhance nuclear NF- κ B activity in T-ALL cells as quantified by EMSA. All these observations strongly suggest that *Hes1* sustains IKK complex activation and maintains the tumor phenotype in T-ALL by directly repressing *CYLD* expression and function.

Targeted IKK *in vitro* silencing affects the growth of human T-ALL lines

Our data so far suggest that targeting the IKK complex could be effective in the treatment of T-ALL. We have shown previously that the proteasome inhibitor Bortezomib could affect T-ALL cell line growth (Vilimas et al., 2007). As Bortezomib is not a specific NF- κ B inhibitor (Hideshima et al., 2009), we have now tested the effects of a cell permeable peptide (NEMO BINDING DOMAIN) that specifically blocks IKK by disrupting the interaction of NEMO to IKK β (May et al., 2000) on several (total of 9) T-ALL cell lines which have present an active Notch pathway and exhibit NF- κ B activation. These human T-ALL cells lines harbour activating Notch1 mutations. As shown in Figure 7A, the NBD peptide was able to efficiently inhibit nuclear NF κ B binding activity in T-ALL lines. Interestingly, silencing NF- κ B led to specific biological effects, as shown by the rapid induction (24h) of apoptosis as determined by Annexin-V binding and caspase-3 activation (Figures 7B,C). No effects on cell cycle

progression were noted suggesting that IKK inactivation is predominantly affecting cell survival (not shown).

To better understand the molecular effects of IKK inhibition *in vitro* we used these five T-ALL lines to study transcriptome changes 16h after the addition of the NBD peptide. The initial conclusion from these experiments is that although the response to NBD treatment was uniform, gene expression changes were both general and cell-line specific (Figure 7D and Figure S7A). All five cell lines showed signs of induction of cell death characterized by the upregulation of *Bcl2L11* (*Bim*), *Bik* and *Foxo3*, in agreement with their apoptotic phenotype, and an inhibition of NF- κ B target genes (such as *Myc*, *Ptpn6*, *Pnkp* and *Gpx1*). Interestingly, another uniform target of IKK inhibition was the Notch pathway itself (including *Hes1*, *Hes4*, *Notch1*, *Notch3*, *Dtx1*) suggesting a mechanism of feedback inhibition. Using qRT-PCR we have verified the NBD-dependent regulation of several of these genes (Figure S7B). These findings suggested that IKK signalling was essential for disease maintenance and proposed that IKK inhibition could be a potential therapeutic tool in T-ALL.

IKK complex activity is essential for the *in vivo* maintenance of T-ALL

One of the key questions in cancer treatment is whether therapeutic targeting of a single signaling pathway is sufficient to affect tumor maintenance and progression. To address this key question *in vivo* we combined the Notch-dependent leukemia model with an inducible IKK γ (Nemo) deficiency (*Mx1-cre⁺Nemo^{fl/fl}*) model. Bone marrow stem and progenitor cells purified from Mx1-cre-inducible NEMO-deficient mice were transduced with Δ EN1-luciferase/ or EGFP-expressing retroviruses and transplanted into irradiated recipients. Two weeks post-transplantation, once the disease had been established as indicated the presence of double CD4⁺CD8⁺ lymphocytes in the peripheral blood (Figure 8A,B) and the expansion of the donor cells as detected by *in vivo* bioluminescence (Figure 8C, D), Nemo expression (and thus canonical NF- κ B activation) was silenced by inducing the expression of cre-recombinase using polyI-polyC injections (Kuhn et al., 1995). As shown in Figures 8B–F, deletion of Nemo very efficiently reverted disease progression as judged by both the significant decrease in luminescence (Fig 8C, D) and the disappearance of transformed CD4⁺CD8⁺ T cells from the peripheral blood of the treated animals (Figure 8A,B) and T-cell infiltration in the spleen and liver (Figure 8F). In agreement to the effects of IKK inhibition on T-ALL cell lines, IKK/Nemo deletion lead to rapid induction of leukemic cell apoptosis (Figure 8E).

Consistent with these phenotypic observations, pI-pC treated *Nemo^{wt/wt}*, *MxCre⁺*-expressing animals succumbed significantly faster than their pI-pC treated *Nemo^{fl/fl}*, *MxCre⁺* counterparts. However, if the treatment was not continuous, the disease was able to relapse and eventually kill the animals (Figure 8G). To determine whether the reappearance of the disease was due to the expansion of a small cell population in which *Nemo* was not deleted, we sorted leukemic cells, 61 days post transplant -approximately 40 days after the last pI-pC injection and tested them for the presence of Nemo. Further supporting the essential function of IKK signaling in T-ALL leukemias, the purified cells were bearing the “undeleted” NEMO allele (Figure S8A) and showed a significant increase of NF- κ B DNA-binding activity in the nucleus (Figure S8B). These studies are the first to demonstrate that IKK activity is essential for the maintenance of T-ALL and open the way for inhibiting the IKK complex as a therapeutic target for patients with already established T-ALL.

DISCUSSION

Our studies demonstrate that the transcriptional repressor Hes1, a target of oncogenic Notch1, is able to induce the activation of the NF- κ B pathway in human T-ALL lines and animal models of the disease. Moreover, they show that IKK/NF- κ B signaling is essential for the maintenance of the disease, as T-ALL cells that are unable to activate the IKK kinase complex rapidly enter

apoptosis. As Notch1 mutations or pathway hyper-activation are found in the vast majority of T-ALL patients, our findings are clinically important as they could be translated to either as single agent therapy targeting IKK (or NF- κ B) activation or combinatorial drug treatments that could in parallel target additional elements of the Notch pathway.

The presented studies identify a previously unidentified mechanism of Notch-induced NF- κ B activation in T-ALL. We show that *Hes1* is a key mediator of Notch-induced transformation by targeting the deubiquitinase *CYLD*, a negative regulator of IKK activity. Our experiments demonstrate that *Hes1* expression is sufficient to enhance NF- κ B activity and this effect maps upstream of I κ B α degradation, IKK phosphorylation, and TAK1 activity. A significant portion of these effects can be attributed to the suppression of *CYLD* expression by *Hes1*. In agreement with this notion, we have identified *CYLD* as a direct target for *Hes1* repression. *CYLD* is a deubiquitinating enzyme that was originally identified as a tumor suppressor and is mutated in familial cylindromatosis (Bignell et al., 2000). More recently, *CYLD* inhibition has been associated with other tumors such as melanoma (Massoumi et al., 2009) and breast (Hutti et al., 2009). Although previous to this report, there was no information about an involvement of *CYLD* in leukemia, abnormal lymphopoiesis in *CYLD*-deficient animals had been reported (Reiley et al., 2006). This abnormal differentiation observed in the *CYLD*-deficient mice was initially associated with a defect in Lck kinase activation in response to TCR signaling, however it was later demonstrated that *CYLD* is also responsible for TAK1 deubiquitination resulting in the inactivation of the NF- κ B pathway in T-lymphocytes (Reiley et al., 2007). Other well-characterized *CYLD* substrates that participate in the NF- κ B signaling include TRAF2, Bcl3 and IKK γ /NEMO. We demonstrate here that *CYLD* expression is down-regulated in T-ALL cell lines and primary samples, and that knocking down *Hes1* expression in these cells results in increased *CYLD* levels and attenuation of the NF- κ B signaling. Interestingly, *Hes1*-mediated suppression appears to be the preferred mode of regulation as no inactivating mutations or *CYLD* promoter hyper-methylation were detected in human T-ALL (data not shown and Figure 6). We should also mention that *CYLD* suppression of expression is by itself unlikely to account for the initial NF- κ B activation. Indeed, it is likely that basal NF- κ B stimulation is required and is provided by either Notch itself (Vilimas et al., 2007) or/and the T cell receptor (TCR). We have previously shown that T-ALL cells express the pre-TCR that can activate constitutively the NF- κ B pathway (Aifantis et al., 2001; Davis et al., 2001; Mandal et al., 2005). It is thus conceivable that the tumors have basal NF- κ B activation and suppression of the negative regulator of the IKK complex (*CYLD*) further boosts pathway activation.

Another important point made by our studies is that Notch/*Hes1* activation is targeting the canonical NF- κ B pathway in T-ALL. NEMO is a regulatory subunit of IKK complex, which is responsible for activation of canonical NF- κ B signaling pathway. The T lineage-specific deletion of NEMO prevents the generation of peripheral T cells due to apoptotic death mature thymocyte (Schmidt-Suppran et al., 2003). Consistent with this notion, silencing NEMO/IKK γ was sufficient to inhibit NF- κ B activation in our animal model of ICN1-dependent T-ALL and affect tumor growth and animal survival. Explaining these effects, gene expression studies in IKK-inactivated T-ALL cells suggested that genes involved in regulating cell survival or apoptosis are targeted by canonical NF- κ B signaling in T-ALL. Although several survival genes have been shown to be NF- κ B targets, two of them, the anti-apoptotic *Bcl2A1* and the pro-apoptotic *Bcl2L11* (*Bim*), both members of the BCL2 family, showed contrasting modes of regulation in response to IKK silencing. *Bcl2A1* appears to play a key role in Notch-induced T-ALL. We have previously shown that *Bcl2A1* is over-expressed in T-ALL and that its silencing was able to affect the survival of both Notch1 and Notch3 over-expressing T cell leukemia lines (Mandal et al., 2005). We have also previously shown that *Bim* is expressed in low amounts in T cell leukemia and that it controls early T cell death induction (Mandal et al., 2008), making this molecule a putative therapy target.

Although the role of NF- κ B in leukemogenesis has been previously reported (Davis et al., 2001), several recent studies in tumors as diverse as Diffuse Large B-cell lymphoma (DLBCL) (Compagno et al., 2009; Kato et al., 2009) or lung adenocarcinoma (Compagno et al., 2009; Kato et al., 2009; Meylan et al., 2009) suggest that the possibility to target this pathway in cancer therapy should be re-evaluated. In agreement with our findings, alterations in different elements of the NF κ B pathway, including the enzymes A20 and CYLD, were found to be responsible for activating NF- κ B in multiple myeloma and DLBCL, upstream of the IKK complex (Annunziata et al., 2007; Compagno et al., 2009; Kato et al., 2009; Keats et al., 2007; Lenz et al., 2008). In lung cancer cells with mutated K-ras, NF- κ B is activated by the non-canonical TBK1/IKK interaction (Barbie et al., 2009) and blocking IKK activity reduces tumor growth *in vivo* (Meylan et al., 2009). We demonstrate here that in T-ALL, IKK can be activated by the Notch-Hes1-CYLD signaling cascade. However, whatever the trigger, the outcome is identical: activation of IKK, degradation of I κ B α and nuclear translocation of the NF- κ B factor, which provides the base to consider that pharmacological targeting of the IKK/NF- κ B pathway could be a promising future treatment of a wide spectrum of tumors. The recent development of chemical inhibitors of the IKK complex, drugs that are currently in clinical trials for inflammatory disease, will enable us to test this hypothesis in future treatment protocols.

EXPERIMENTAL PROCEDURES

Mice and Animal procedures

C5/BL/6, or BALB/C mice were purchased from Jackson Laboratories and Taconic Farms. BALB/C-Tg(NF- κ B-RE-luc [Oslo]) were purchased from Caliper. *Nemo*^{fl/f} mice have been described previously (Schmidt-Supprian et al., 2000). *Cyld* knockout mice were generated as described previously (Reiley et al., 2007). For the polyI-polyC experiments, we injected 20 μ g pI-pC (Amersham) per gram of body weight. All mice were kept in specific pathogen-free animal facilities. All animal procedures were approved by and performed in accordance to the guidelines of the Institutional Animal Care and Use Committee of the NYU-SOM.

Human T-ALL samples

A total of 33 T-ALL samples and normal blood samples were obtained from the Hematology Department of Hospital de Sant Pau and the Department of Pathology of Hospital del Mar, Barcelona, Spain. All patients gave written consent to donate the blood specimen. The study was approved by the Ethics Committee of the Institution (Department of Pathology, Hospital del Mar).

Plasmids

3x κ B-luciferase and HA-I κ B α _{S32-36A} (Espinosa et al., 2003), I κ B α K21-22R (Arenzana-Seisdedos et al., 1997), HA-TAK1K63W (Ninomiya-Tsuji et al., 1999), Δ EN1-Luciferase, Δ EN1-GFP and ICN1-GFP retroviral constructs (Chiang et al., 2006; Vilimas et al., 2007) or, ICN1 Δ OP (Bigas et al., 1998) have been previously described. Flag-Hes1 was obtained from M. Caudy (Castella et al., 1999) and used to clone Hes1 cDNA into the inducible vector pcDNA3.TO. pTHR1-NF- κ B-dscGFP and pTHR1-mCMV-dscGFP lentiviral constructs were purchased from System Biosciences. CYLD retroviral and shRNA constructs were given by A. Pfeifer (Massoumi et al., 2009). Human and mouse-specific *Hes1*-shRNA lentiviral constructs was purchased from Sigma (TRCN0000018989 and TRCN0000028854 respectively).

Bioluminescence imaging

Imaging of κ B-ER-luciferase and Δ EN1-luciferase chimeras were performed as previously described (Tseng et al., 2006).

qRT-PCR and Microarray

The primers used for RT-PCR analysis are listed in Supplemental Experimental Procedures. qRT-PCR was performed in LightCycler480 system using SYBR Green I Master or Probes Master kit (Roche). For microarray analysis, the dataset of the genome-wide RNA expression analyses of human T-ALL cells lines treated by control peptide versus NBD peptide has been deposited in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>); the accession numbers is GSE20667. RNA was labeled on hybridized to the Human U133 Plus 2.0 microarray (Affymetrix). The Affimetrix gene expression profiling data was first normalized using previously published Robust Multi-array Average (RMA) algorithm implemented in RNAExpress software version 1.0.4 (<http://rmaexpress.bmbolstad.com/>) and log2 transformed. Then genes that are differentially expressed between NBD peptide treated and control peptide treated cells were selected using a paired two-side t-test and requiring fold change ≥ 1.2 or ≤ 0.833 . The multiple comparison correction was performed using the approach of (Reiner et al., 2003) with FDR threshold equal to 0.10. The application of the above procedure resulted in 387 genes (531 oligonucleotide probes).

Quantitative DNA methylation analysis

DNA methylation analysis was carried out using the EpiTyper system from Sequenom (San Diego, CA). The EpiTYPER assay is a tool for the detection and quantitative analysis of DNA methylation using base-specific cleavage of bisulfite-treated DNA and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Specific PCR primers for bisulfite-converted DNA were designed using the EpiDesigner software (www.epidesigner.com), for the entire CpG island of the genes of interest. T7-promoter tags are added to the reverse primer to obtain a product that can be *in vitro* transcribed, and a 10-mere tag is added to the forward primer to balance the PCR conditions. Primer sequences, target chromosomal sequence, and EpiTyper specific tags, are available upon request. A detailed description of the protocol used can be found at the Supplemental Experimental Procedures.

Statistical analysis

Student's T-test was performed to assess the significance of expression levels. These data are presented as mean $\pm$ SD.

SIGNIFICANCE

Although aberrant activation of the Notch pathway is a recurring event in T-ALL, the downstream signaling effects of this activation are poorly defined. Our work demonstrates that the Notch-Hes1-CYLD-IKK axis plays a crucial role in this type of leukemia, and that blocking NF- κ B (or the IKK kinase) is sufficient to eliminate leukemic cells carrying activating Notch mutations. These findings suggest that NF- κ B targeting could be a key component of successful future T-ALL clinical trials.

HIGHLIGHTS

NF- κ B activity is active and essential in established Notch-dependent T-ALL leukemias. Notch through Hes1 sustains NF- κ B activity by repressing the deubiquitinase CYLD. Inhibition of IKK/NF κ B activity reduces leukemic load and increases survival in mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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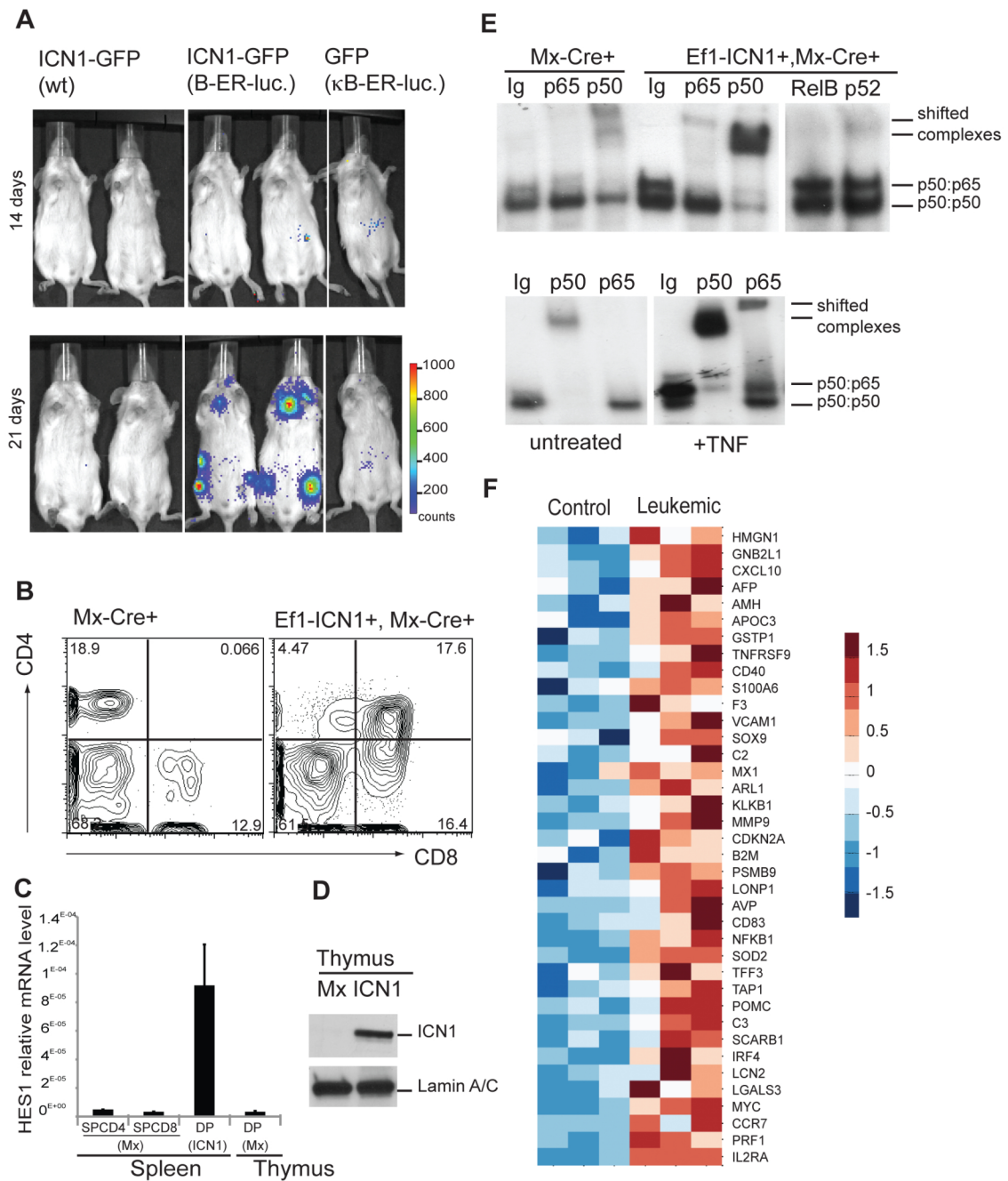


Figure 1. Oncogenic Notch induces NF-κB activation in T-ALL animal models

(A) NF-κB luciferase reporter activity was detected in mice transplanted with: ICN1-GFP (WT), ICN1-GFP (NFκB-ER-luc) or GFP (NFκB-ER-luc). Bioluminescent imaging of mice 9 and 21 days after transplantation is shown. (B) Peripheral blood CD4/CD8 FACS analysis; (C) qPCR from sorted cells as indicated. Error bars denote $\pm$ Standard Deviation. (D) Protein levels of Notch1-IC (ICN1) and Lamin A/C in nuclear extracts of thymocytes from Mx-Cre (Mx) and Ef1α-ICN1, Mx-Cre (ICN1) mice 4 weeks after pIpC injection. (E) NF-κB DNA binding activity was analyzed by EMSA using nuclear extracts of thymocytes from MxCre, Ef1α-ICN1 Mx-Cre mice 4 weeks after pIpC injection and wt thymocytes either untreated (Co) or treated with mTNFα. (F) Heat-map shows expression of NF-κB target genes selected from

the literature (<http://www.nf-kb.org>) up-regulated in leukemic DP cells compared to control thymic DP cells by unpaired one-side t-test with $\alpha=0.05$. See also related figure S1.

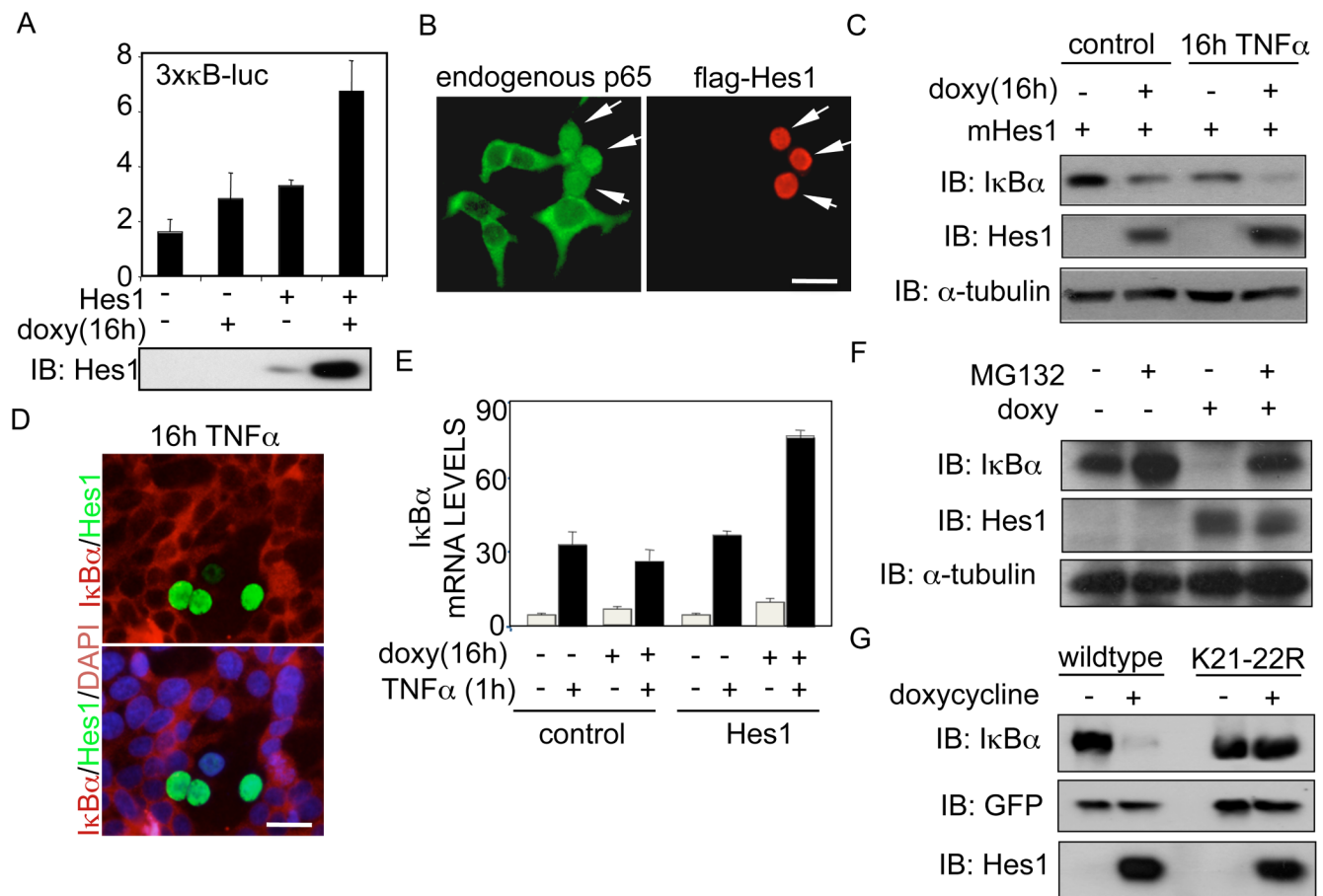


Figure 2. Hes1 enhances NF- κ B-dependent activity by inducing I κ B α degradation

(A) Luciferase assay with the 3 κ B-luc-reporter in HEK-293T cells transfected with a doxycycline-inducible Hes1 construct. Induction of Hes1 after doxycycline treatment is shown in the lower panel. (B) Immunofluorescence of HEK-293T transfected with flag-Hes1 showing the expression pattern of transfected Hes1 and endogenous p65. (C) Western blot of endogenous I κ B α in HEK-293T cells transfected with inducible Hes1 in the presence or absence of TNF α . (D) Lack of I κ B α immunostaining in cells transfected with ectopic Hes1. (E) Quantitative RT-PCR to determine the expression levels of I κ B α mRNA in the presence or absence of Hes1. Error bars denote $\pm$ Standard Deviation. (F) Western blot of I κ B α levels in HEK-293T transfected with the doxycycline-inducible Hes1 construct in the presence or absence of the proteasome inhibitor MG132. (G) Western blot showing the levels of I κ B α wt or I κ B α _{K21-22R} cotransfected with inducible Hes1. See also related figure S2.

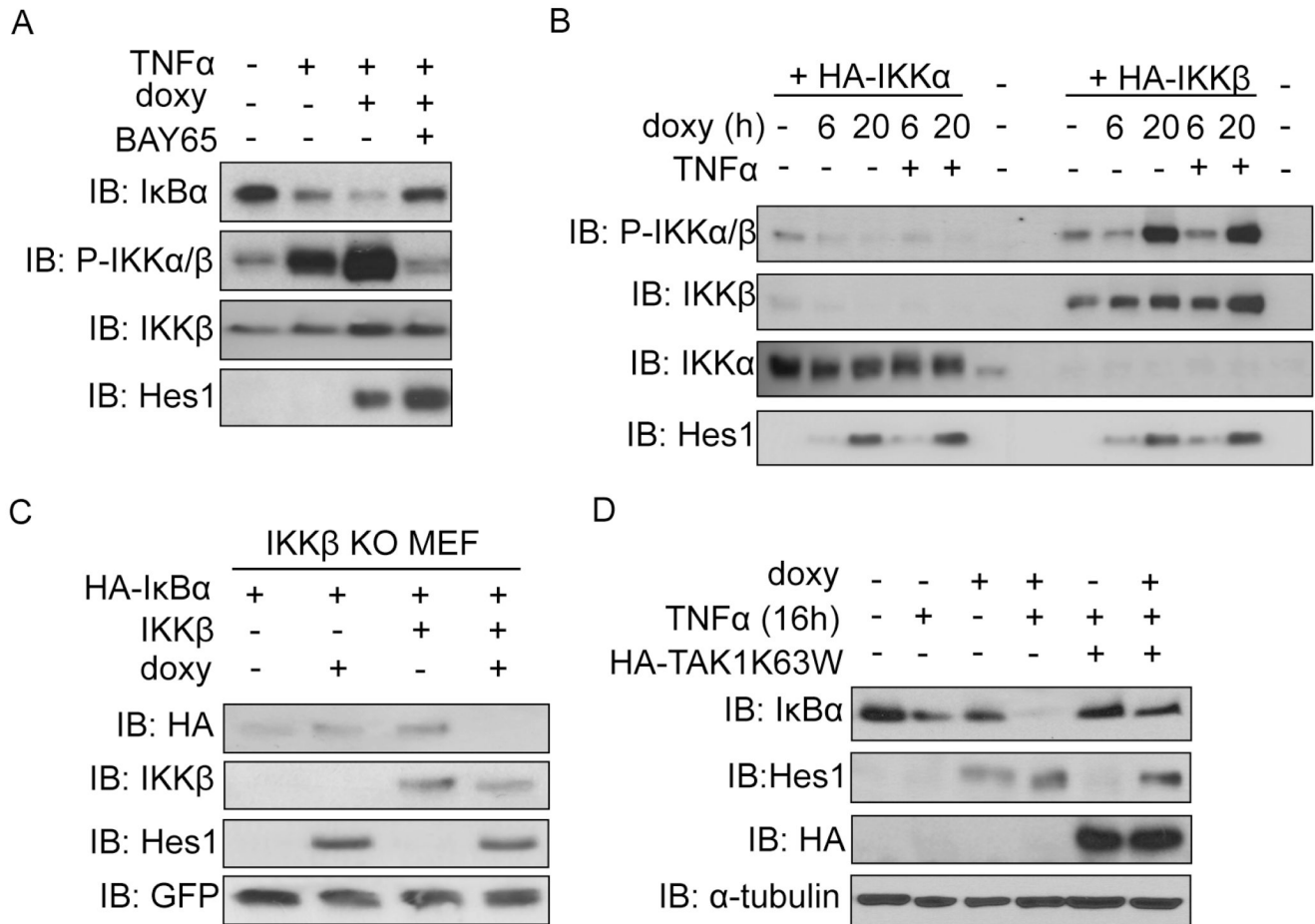


Figure 3. Hes1 facilitates I κ B α degradation by regulating IKK β activity

Western blots of cells transfected with an inducible Hes1 vector and treated for 16h with doxycycline and TNF α as indicated. (A) HEK-293T cells were treated with the IKK inhibitor BAY65-1185, (B) Cells were cotransfected with HA-IKK α or HA-IKK β , (C) IKK β -deficient cells were reconstituted with HA-IKK β and (D) HEK-293T cells were transfected with the dominant negative TAK1 (K63W) construct. See also related figure S3.

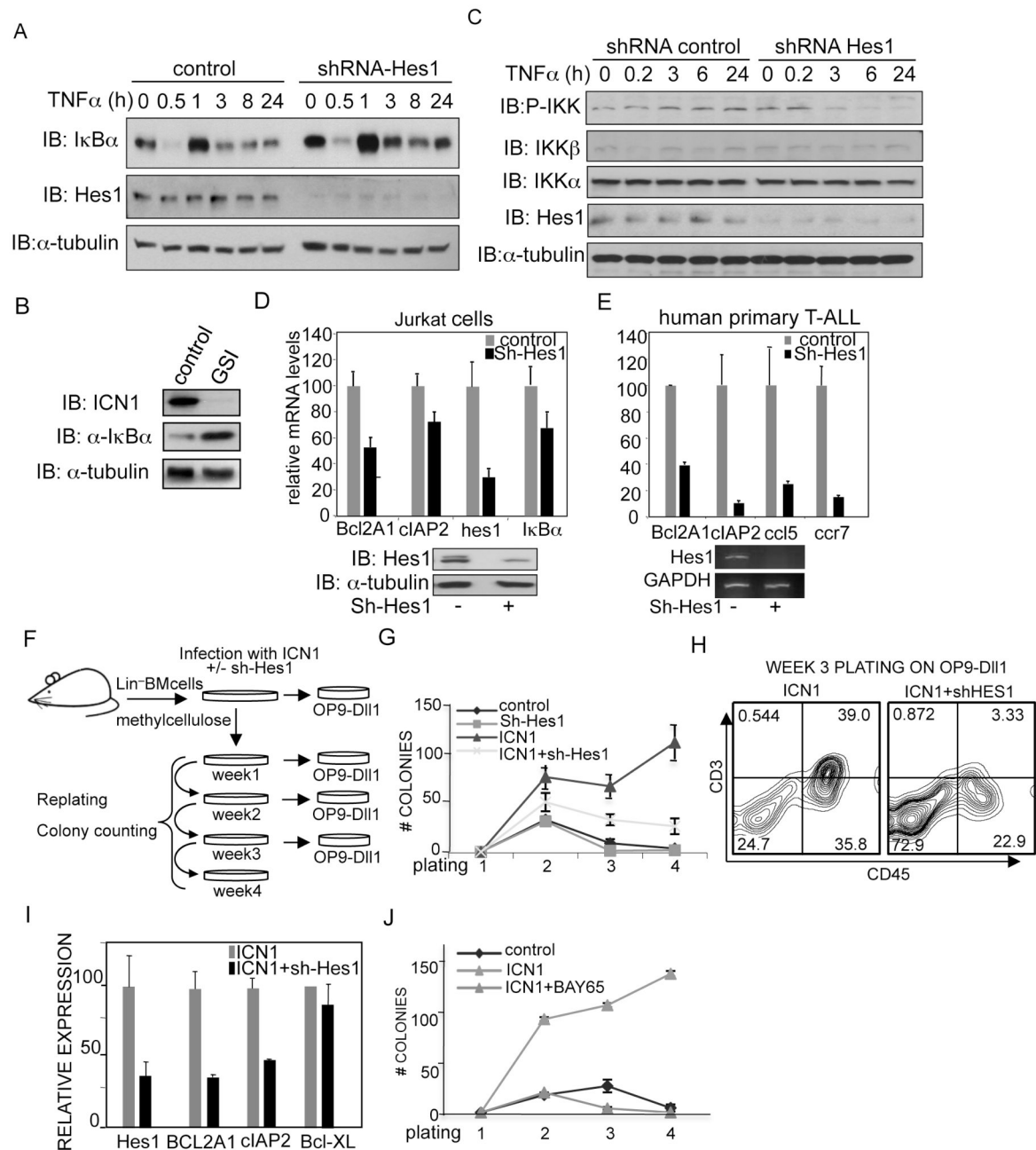


Figure 4. Inhibition of Hes1 in cells with active Notch1 prevents NF-κB activity leading to increased apoptosis

(A) Jurkat cells transduced with scrambled shRNA or *Hes1*-shRNA (80 to 90% reduction in Hes1 protein levels) were analyzed for the levels of IκBα protein during TNFα treatment, (B) IκBα is restored in the presence of GSI (L685,458) (C) levels of P-IKK in control and Hes1 knockdown cells by western blot and (D) expression of NF-κB-target genes by qRT-PCR in Jurkat or (E) primary human T-ALL leukemic cells transduced with the control or *Hes1*-shRNA lentivirus. (F) Experimental design to test the contribution of Hes1 to ICN1 transduced cells. (G–I) Primary mouse Lin⁻ bone marrow progenitors transduced with the indicated lentivirus. (G) Colonies obtained from serial replatings on methylcellulose were counted. (H) T-cell

differentiation was determined by CD3 expression after 7 days in co-culture on OP9-Dll1. One representative experiments out of three is shown. (I) Expression levels of the indicated genes were tested by qRT-PCR after 2 weeks in culture. (J) Number of colonies obtained in serial plating from ICN1 transduced cells in the presence or absence of BAY65-5811. Error bars denote $\pm$ Standard Deviation. See also related figure S4.

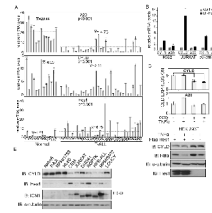


Figure 5. The expression of CYLD, a negative regulator of NF- κ B, is suppressed in T-ALL
 (A) Levels of *A20*, *CYLD* and *Hes1* mRNA in primary samples for normal WPB (1–9), purified normal CD3⁺ cells from PB (10–13) and PB from T-ALL patients (1–33) by qRT-PCR. (B) Analysis by qRT-PCR of *CYLD* or *A20* levels in cells transduced with scrambled or *Hes1*-shRNA. For both A–B panels, error bars denote $\pm$ Standard Deviation. (C–D) Expression of *CYLD* and *A20* measured by qRT-PCR (C) or western blot (D) in HEK-293T cells expressing ectopic *Hes1* untreated or treated with TNF α . (E) Western blot analysis of *CYLD*, active Notch1 and *Hes1* protein levels in different leukemic cell lines. See also related figure S5.

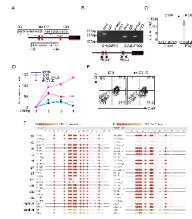


Figure 6. Hes1 directly represses CYLD transcription in T-ALL

(A) Scheme of the *CYLD* promoter indicating the putative Hes1 binding sites. (B) Detection of the *CYLD* PRO-2 Hes1 binding site by PCR using the indicated antibodies. (C) Relative enrichment of the *CYLD* promoter quantified by qPCR from ChIP with anti-Hes1 antibody in Jurkat T-ALL cells transduced with scrambled or *Hes1*-shRNA. Error bars denote $\pm$ Standard Deviation. (D) Primary mouse Lineage-negative bone marrow progenitors were transduced with the indicated lentivirus and colonies obtained from serial replating on methylcellulose were counted every week. (E) Representative FACS analysis from week 3 progenitors after 7 days on OP9-Dll1 stroma. (F) EPITYPER methylation analysis showing no significant hypermethylation of the *CYLD* promoter. Patient samples were analyzed utilizing the EpiTYPER Sequenom methylation assay. The heat map illustrates the range of methylation percentages, with red indicating 0% methylation and yellow indicating 100% methylation. Numbers along the top indicate CpG dinucleotide, patient sample numbers are listed along the vertical axis (p denotes the identity of the patient as presented in Figure 5A). Samples are run in duplicate. Two primers were run for each region. The positive control, IVD, has near 100% methylation, while the negative control, WGA, is unmethylated. See also related figure S6.

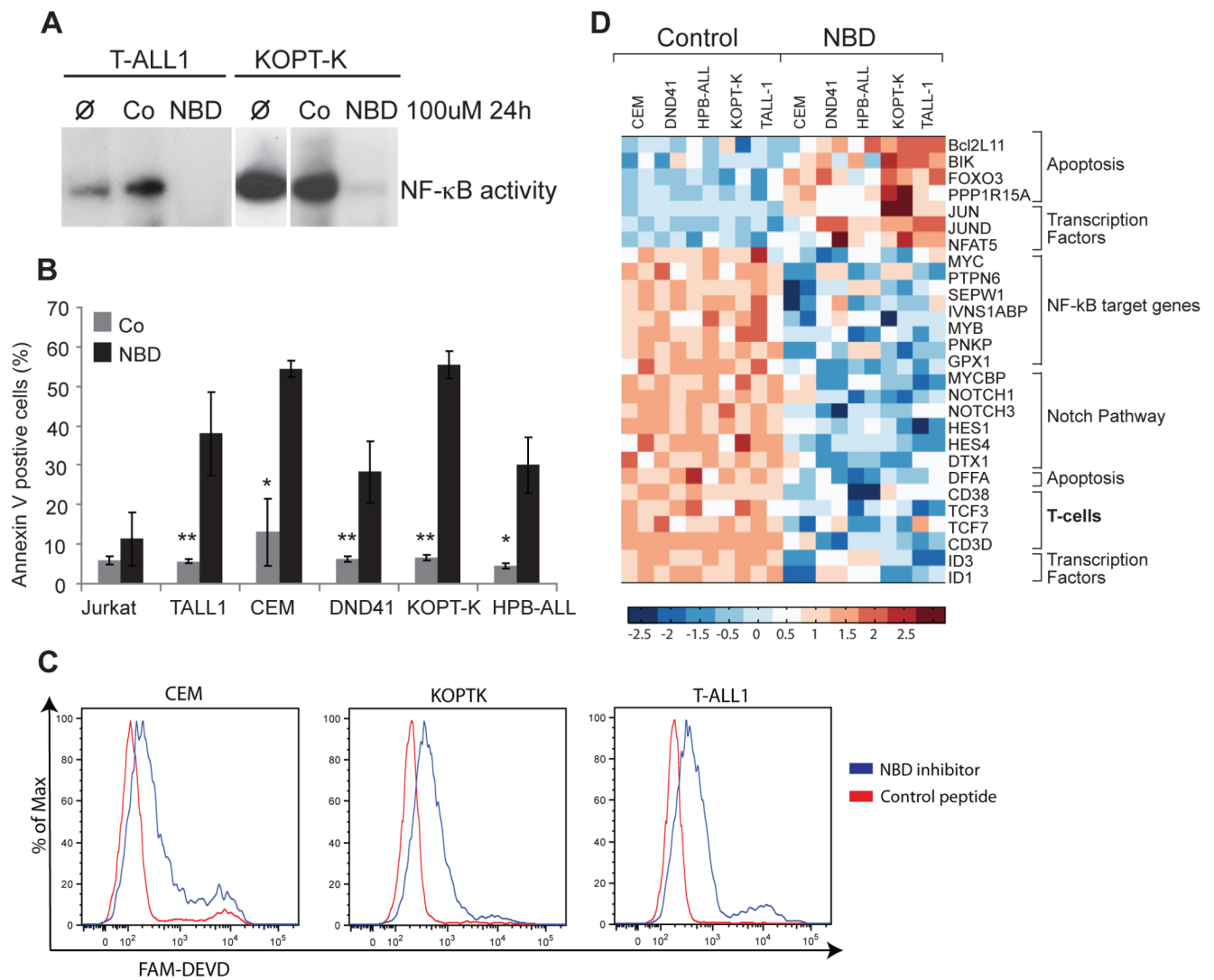


Figure 7. IKK *in vitro* silencing affects the survival of human T-ALL cell lines

Human T-ALL cell lines were treated with 100 μ M of control (Co) or NBD inhibitory peptide for 24 hours. (A) Nuclear extract from treated and untreated cells were isolated and checked for NF- κ B-DNA binding activity by EMSA. Untreated cells (Ø) are also shown. (B) Bar-graph indicating the proportion of Annexin V positive cells 24h after the NBD treatment. Error bars denote $\pm$ Standard Deviation. (C) Caspase-3 activation as detected by FACS with fluorogenic cell permeable active caspase 3- specific probe (Fam-DEVD-fmk) in 3 representatives T-ALL lines 24h post treatment. All experiments shown in A–C were repeated at least three times. (mean $\pm$ SD of measurements: *, $p < 0.05$; **, $p < 0.01$ Student's *t* test analysis). (D) Heat map showing up changes in expression of selected genes in response to NBD peptide treatment. See also related figure S7.

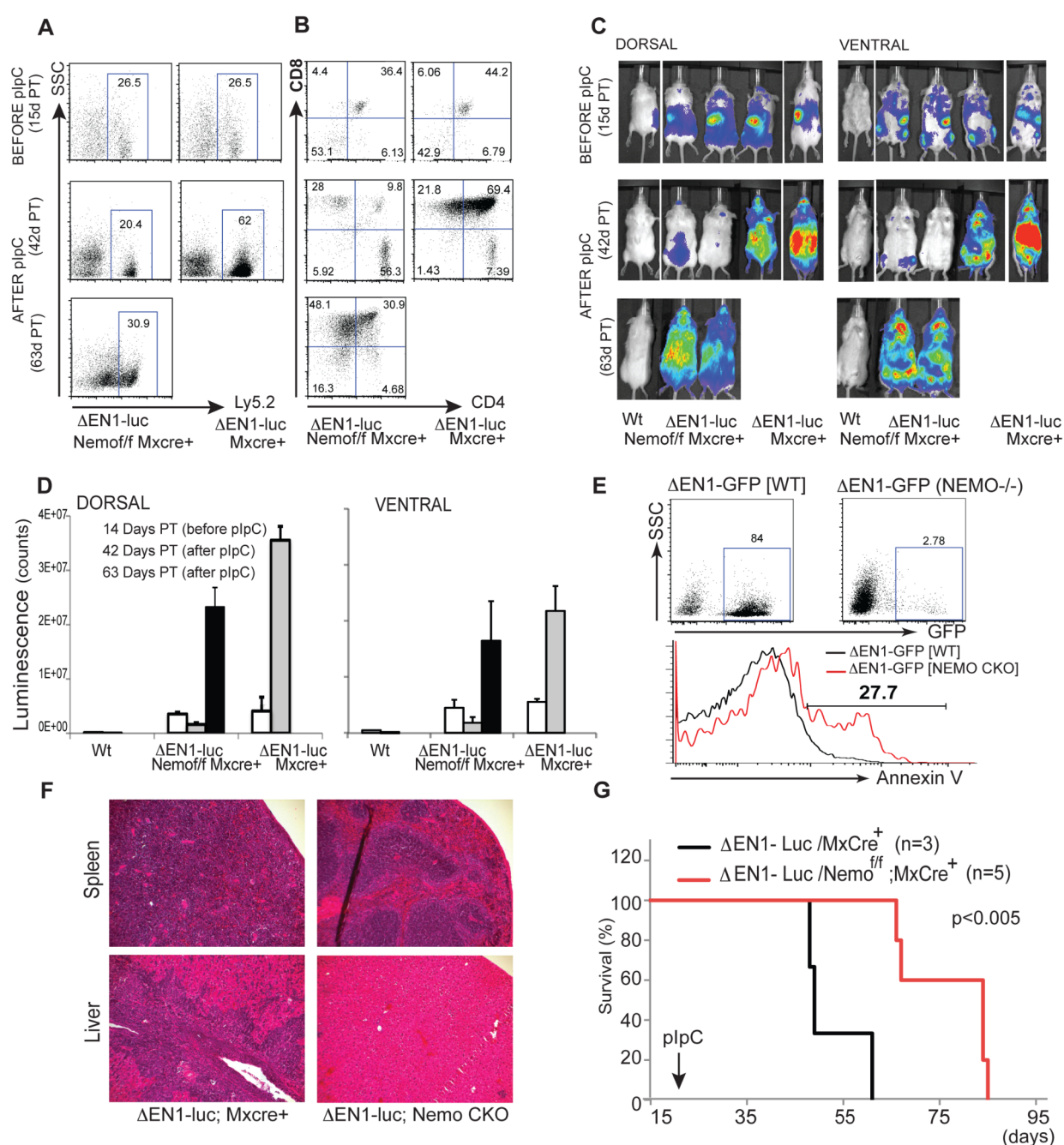


Figure 8. The NEMO/IKK complex activity is essential for the maintenance of T-ALL in vivo (A–B) Peripheral blood FACS analysis using CD4, CD8 and Ly5.2 antibodies at different time points post transplantation. (C) $\Delta EN1$ -luc $Nemo^{wt/wt}$; Mx-Cre and $\Delta EN1$ -luc $Nemo^{ff/ff}$; Mx-Cre chimera were monitored before and after pIpC injections using *in vivo* imaging system (IVIS) to quantify luciferase intensity. 2 weeks post-transplantation and before any deletion of NEMO, both chimera harbored homogenous tumor load. Once this was verified mice were injected by pIpC. (D) Quantification of luciferase intensity (dorsal and ventral) at different time-points. Error bars denote $\pm$ Standard Deviation. (E) Determination of apoptotic cells gated on GFP⁺ cells 5 days after pIpC injections in $\Delta EN1$ -GFP $Nemo^{wt/wt}$; Mx-Cre and $\Delta EN1$ -GFP $Nemo^{ff/ff}$; Mx-Cre chimera. (F) Micrographs of hematoxylin/eosin-stained liver and

spleen sections prepared 40 days after transplantation. (G) Kaplan-Meyer survival curve of the different recipient animals ($p < 0.005$). See also related figure S8.