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Functional loss of IkBε leads to NF-κB deregulation in aggressive chronic lymphocytic leukemia

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NF-κB is constitutively activated in chronic lymphocytic leukemia (CLL); however, the implicated molecular mechanisms remain largely unknown. Thus, we performed targeted deep sequencing of 18 core complex genes within the NF-κB pathway in a discovery and validation CLL cohort totaling 315 cases. The most frequently mutated gene was NFKBIE (21/315 cases; 7%), which encodes IkBε, a negative regulator of NF-κB in normal B cells. Strikingly, 13 of these cases carried an identical 4-bp frameshift deletion, resulting in a truncated protein. Screening of an additional 377 CLL cases revealed that NFKBIE aberrations predominated in poor-prognostic patients and were associated with inferior outcome. Minor subclones and/or clonal evolution were also observed, thus potentially linking this recurrent event to disease progression. Compared with wild-type patients, NFKBIE-deleted cases showed reduced IkBε protein levels and decreased p65 inhibition, along with increased phosphorylation and nuclear translocation of p65. Considering the central role of B cell receptor (BcR) signaling in CLL pathobiology, it is notable that IkBε loss was enriched in aggressive cases with distinctive stereotyped BcR, likely contributing to their poor prognosis, and leading to an altered response to BcR inhibitors. Because NFKBIE deletions were observed in several other B cell lymphomas, our findings suggest a novel common mechanism of NF-κB deregulation during lymphomagenesis.

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Consisting of five members, NFKB1 (p50), NFKB2 (p52), RELA (p65), RELB, and c-REI (REL), the NF-κB signaling pathway regulates many cellular processes, including cell cycle progression, differentiation, and apoptosis (Bonizzi and Karin, 2004). These proteins form homo- and heterodimers that are held in the cytoplasm by inhibitor proteins (IκB) and function by activating or suppressing target genes (Bonizzi and Karin, 2004). The IκBs (α, β, δ, ε, and θ) are regulated by the IκB kinase complex, which, when activated, phosphorylates the IκBs, leading to their degradation; this culminates in the translocation of transcription factors to the nucleus. In B cells, the canonical NF-κB pathway can be activated through numerous upstream signals including B cell receptor (BcR) or TLR signaling, whereas the noncanonical pathway is primarily activated through BAFF receptor–CD40 interaction (Bonizzi and Karin, 2004; Hömig-Hölzel et al., 2008).

Deregulated NF-κB signaling appears to be particularly important in B cell malignancies, with recurrent activating mutations identified in both the canonical and the noncanonical NF-κB pathways (Compagno et al., 2009; Staudt, 2010; Rossi et al., 2013a). In chronic lymphocytic leukemia (CLL), NF-κB activation is known to be present in virtually all cases (Hershman et al., 2011). That notwithstanding, the extent to which genetic aberrations contribute to NF-κB activation in CLL remains largely unknown except for low-frequency (<3%) mutations in BIRC3 (noncanonical NF-κB pathway) and MYD88 (TLR signaling; Baliakas et al., 2015). Very recently, a recurrent 4-bp truncating mutation within the NFKBIE gene, which encodes IκBe, a negative regulator of NF-κB in B cells, has been reported as frequent in advanced stage CLL (Damm et al., 2014). However, the precise functional impact of this mutation and, especially, the extent to which it contributes to constitutive NF-κB activation in CLL remain unexplored.

To gain insight into these issues, we undertook a combined genetic and functional approach for investigating the NF-κB signaling pathway in CLL. Taking advantage of HaloPlex technology (Agilent Technologies), we designed a targeted gene panel and performed deep sequencing of 18 members of the NF-κB pathway in 315 CLL cases. The most striking observation was the finding of the recurrent frameshift deletion within the NFKBIE gene, which resulted in profound functional consequences. In particular, patients carrying this truncating mutation displayed lower IκBe expression and reduced IκBe–p56 interactions, as well as increased levels of phosphorylated p65 and nuclear p50/p65. Because we also detected this truncating event in other lymphoma entities, our finding implies that the loss of IκBe may be a common mechanism contributing to the sustained survival of malignant B cells, thus also shaping disease evolution and ultimately impacting disease progression.

RESULTS AND DISCUSSION
Targeted sequencing identifies NFKBIE mutations as a recurrent event in CLL

We performed targeted deep sequencing of 18 NF-κB core complex genes (Table S1) within a discovery cohort of 124 CLL patients (Table S2). Sequencing resulted in a mean read depth of 656 reads/base and 97% of the targeted coding regions being covered (Table S1). By applying a conservative cutoff of >10% for the mutant allele, we identified 26 mutations in 11/18 NF-κB genes analyzed within 24/124 (19%) CLL patients (Table S3); 16/16 selected mutations were validated by Sanger sequencing. IκBe (encoded by NFKBIE) was the most frequently mutated, being altered in eight patients (6.5%); notably, three/eight patients carried an identical 4-bp frameshift deletion in NFKBIE exon 1 (Fig. 1 A). When considering mutations with a low mutant allele frequency (<10%), this 4-bp deletion within NFKBIE was found in eight additional cases (Table S4).

NFKBIE mutations predominated in CLL cases with unmutated Ig heavy variable (IGHV) genes (U-CLL) belonging to certain subsets with restricted BcR Igs (stereotyped BcRs), for which we and others have reported distinct, subset-based profiles regarding their biological background and clinical course (Stamatopoulos et al., 2007; Agathangelidis et al., 2012; Strefford et al., 2013; Baliakas et al., 2014). Prompted by this observation, we again performed targeted resequencing of NF-κB genes using HaloPlex technology within a validation CLL cohort (n = 191) enriched for cases assigned to poor-prognostic stereotyped subsets (Tables S5 and S6). We found 30 mutations in 10/18 NF-κB genes analyzed within 28 CLL patients; strikingly, 13/30 mutations were in IκBe with 10/13 patients carrying the 4-bp NFKBIE deletion (Fig. 1 B and Table S7). This deletion was also detected at a low mutant allele frequency (<10%) in 18 additional cases (Table S4).

Because germline DNA was lacking for the vast majority of patients (because of the retrospective nature of the study), we were limited in our ability to confirm the somatic nature of mutations. That said, we could verify that mutations within the NFKBIE gene were somatic and not germline variants in all cases with available material (Tables S3 and S7). For the remaining NF-κB mutations, we cannot formally exclude the possibility that they are rare germline variants (despite extensive filtering against various SNP databases) and hence decided to focus on NFKBIE, which, importantly, was also the most frequently mutated NF-κB gene.

Enrichment of NFKBIE aberrations in poor-prognostic subsets of CLL

We next developed a GeneScan assay specific for the 4-bp NFKBIE deletion and studied 377 additional CLL cases, including (a) patients from a population-based cohort (Table S8), where U-CLL accounted for 32% of cases, with the remaining cases carrying mutated IGHV genes (M-CLL); (b) patients with stage B/C disease; and (c) patients assigned to stereotyped subsets. Overall, 22 additional NFKBIE-deleted patients were identified (Table S9). Collectively, this amounted to 43/692 (6.2%) CLL patients carrying NFKBIE aberrations (i.e., mutations and/or deletions), of whom 37/43 concerned U-CLL. A significant enrichment of NFKBIE aberrations was observed in certain poor-prognostic stereotyped CLL subsets, especially subset #1 (17/112 cases, 15%) and the less
populated subset #6 (5/35 cases, 14%; Fig. 1 B), further supporting the concept that the subclassification of CLL based on BcR stereotypy may supersede the more generic discrimination into U-CLL or M-CLL (Baliakas et al., 2014). A considerably lower frequency of NFKBIE aberrations was observed within our population-based cohort (4/236, 1.7%; Smedby et al., 2005), whereas advanced stage B/C patients carried NFKBIE aberrations at a frequency of 6.1% (12/198), thus lower than recently reported (10%; Damm et al., 2014).

In Fig. 2 A, we depict coexisting cytogenetic/molecular lesions in the 43 patients with NFKBIE aberrations; although a small proportion of cases carried concomitant poor-prognostic TP53 (7%), NOTCH1 (14%), and SF3B1 (9%) mutations, the majority of cases did not carry mutations within these genes. Because mutations have been described in two other NF-κB pathway genes in CLL, MYD88 and BIRC3, albeit at a low frequency (Baliakas et al., 2015), we also sequenced the hotspot p.L265P MYD88 mutation and exons 6–9 of BIRC3. In total, 4/495 (0.8%) patients carried a p.L265P MYD88 mutation, none of which co-occurred with a mutation in NFKBIE, whereas 8/568 (1.4%) patients harbored mutations within BIRC3, with only 1 of these patients carrying the 4-bp deletion within NFKBIE. In addition, we analyzed copy number data for 369 CLL cases obtained from SNP arrays (250K) and found only 3 cases showing a potential monoallelic deletion covering the NFKBIE gene (on chromosome 6p21.1); none of these cases had a truncating NFKBIE mutation (not depicted).

The remarkable enrichment of NFKBIE aberrations in poor-prognostic subset #1 (17/43 NFKBIE-mutated/deleted cases, 39.5%) recalls the significantly higher frequency of SF3B1 mutations in poor-prognostic stereotyped subset #2 compared with all remaining CLL (~44% vs. ~5%; Rossi et al., 2013b; Strefford et al., 2013). This subset-biased distribution of genomic aberrations in different poor-prognostic stereotyped subsets supports the existence of distinct mechanisms underlying clinical aggressiveness in CLL and could perhaps result from particular modes of BcR-mediated signaling, which could shape the evolution of each individual subset. In other words, the enrichment seen in stereotyped subsets might primarily be linked to the particular BcR configuration of each subset rather than merely attributed to IGHV gene mutational status.

**NFKBIE aberrations are linked to rapid disease progression and poor outcome**

The presence of NFKBIE aberrations was associated with a significantly shorter time to first treatment (TTFT) similar to IGHV-unmutated or 17p-deleted patients (Fig. 2 B), which was perhaps expected given the preference toward clinically aggressive CLL subsets (Stamatopoulos et al., 2007; Baliakas et al., 2014). In multivariate analysis including established risk factors, NFKBIE aberrations did not hold as an independent factor; however, when IGHV mutational status (one of the strongest molecular predictors of TTFT in CLL [Baliakas et al., 2015]) was removed from the model, NFKBIE aberrations regained significance (Table S10). Taking into account that almost all cases with NFKBIE aberrations concerned U-CLL, this could be the overarching reason behind this latter finding, along with the comparatively lower number of cases in the NFKBIE-mutated/deleted group (relative to IGHV-unmutated CLL). Despite a limited number of cases showing
poor outcome seen for the vast majority of cases with clinicobiological factor was identified that could explain the aberrations was found to remain stable or essentially unaltered at relapse. This has to be studied in more detail, in particular because the temporal dynamics is indicative of clonal evolution and potentially links these aberrations to disease progression. Admittedly, this has to be studied in more detail, in particular because the variant allele frequency of several cases with low-frequency NFkBIE aberrations was found to remain stable or essentially unaltered at relapse.

Because NFkBIE aberrations were linked to inferior outcome and considering the finding of low-frequency (<10%) 4-bp NFkBIE deletions in a considerable proportion of cases (Table S4), we also investigated longitudinal samples available from 14 treated CLL cases. These cases exhibited varying allele frequencies in the initial sample investigated (8/14 cases <10%, range 1–8%), and an increase in the allelic frequency of the NFkBIE mutations and/or deletions was observed over time and at relapse in 6/14 cases (Fig. 2 C). Such temporal dynamics is indicative of clonal evolution and potentially links these aberrations to disease progression. Admittedly, this has to be studied in more detail, in particular because the variant allele frequency of several cases with low-frequency NFkBIE aberrations was found to remain stable or essentially unaltered at relapse.

Considering our findings in CLL and the sparse reporting of NFkBIE mutations in other lymphomas (Emmerich et al., 2003; Gunawardana et al., 2014), we performed a comprehensive screening of 372 additional mature B cell lymphomas, 3/66 (4.5%) diffuse large B cell lymphomas (DLBCLs), and 3/170 (1.8%) splenic marginal zone lymphomas. These results are highly indicative of a common mechanism for NF-kB deregulation within at least a subset of mature B cell malignancy cases (Fig. 1 B).

IκBɛ disruption results in reduced inhibition and increased nuclear p65 levels

In normal B cells, IκBɛ provides negative regulation upon Bcr/TLR stimulation by limiting nuclear migration of Rel-containing NF-κB dimers (e.g., p65 and REL) through protein binding via the ankyrin repeat region (Fig. 1 A), thus ensuring temporal control of NF-κB activation (Alves et al., 2014). Furthermore, IκBɛ loss was reported to result in increased B cell proliferation and survival of stimulated B cells in IκBɛ−/− mice (Alves et al., 2014). To understand the functional consequence of truncating NFkBIE mutations for the NF-κB signaling pathway in CLL, the three IκB members (α, β, and ε) were investigated together with the transcription factor p65 using Western blot analysis. Significantly lower IκBɛ protein levels were observed in NFkBIE-deleted (n = 7, mean allele frequency 45%, range 28–61%) versus WT patients (n = 7; P < 0.001), whereas no differences were detected for either IκBα or IκBβ (Fig. 3, A–C). Accordingly, phosphorylated p65 levels were significantly higher in NFkBIE-deleted versus WT patients (Fig. 3, A–C; P < 0.05).

The 4-bp frameshift NFkBIE deletion is predicted to lead to the introduction of a stop codon and subsequent loss of the ankyrin repeat region, thus potentially resulting in a truncated form (with a predicted mass of 13.4 kD) lacking...
Using an alternative approach by applying proximity ligation assays, a highly sensitive method for real-time visualization of protein–protein interactions in situ (Söderberg et al., 2006). In six NFKBIE WT CLL cases, although interactions were detected for all IκB in unstimulated CLL cells, IκBα exhibited the greatest number of interactions with p65 per cell analyzed, supporting its important role in CLL (Fig. 4 C). Upon stimulation with αIgM or CD40 ligand (CD40L), although the interactions between all IκB and p65 were reduced, IκBα was predominantly affected (Fig. 4, C–F). In contrast, in six NFKBIE-deleted cases, IκBα and p65 interactions in unstimulated CLL cells were notably reduced, thus resembling stimulated WT cells (Fig. 4, C–F); however, this finding did not reach statistical significance (P = 0.15), probably because of the low number of cases available for analysis.

Altogether, our data indicates that these truncating mutations reduce IκBα levels, in turn leading to reduced IκBα–p65 interactions, and, consequently, increasing phosphorylated p65, which is potentially underlying a more activated state. This was further supported by subsequent fractionation experiments on January 26, 2016 jem.rupress.org Downloaded from Published May 18, 2015

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Figure 3. Protein expression analysis in NFKBIE-deleted versus WT CLL. (A) Protein expression profiles of IκBα, IκBβ and IκBε, p65, and phospho-p65 in NFKBIE-deleted (n = 4) versus WT (n = 4) CLL by Western blot analysis. (B) Protein expression profiles of IκBα, IκBβ and IκBε, p65, and phospho-p65 in additional NFKBIE-deleted (n = 3) versus WT (n = 3) CLL by Western blot analysis. (C) Mean normalized protein expression values for IκBα, IκBβ and IκBε, p65, and phospho-p65 in NFKBIE-deleted (n = 7) versus WT CLL (n = 7). * indicates P < 0.05, whereas ** indicates P < 0.001. For IκBβ, only four NFKBIE-deleted and four WT CLL cases were assessed. Error bars indicate standard error. (D) Western blot analysis of IκBε in CLL to identify the presence of a truncated IκBε protein. The membrane is overexposed. del, deleted.
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45%, range 28–61%) and nine WT patients. Although an interesting up-regulation of several small nuclear RNAs (i.e., SNORD66, SNORD114-1, and SNORA80-B), previously linked to cancer (Gao et al., 2015), was observed in the NFKBIE-deleted group, only a few genes were significantly differentially expressed between the subgroups (Table S14). This finding might reflect previous gene expression profiling studies in U-CLL and M-CLL, showing only subtle differences in gene expression signatures in these clinically distinct subgroups (Klein et al., 2001; Rosenwald et al., 2001); along this line, one gene known to be up-regulated in U-CLL is ZAP70, and this gene also showed a higher expression in NFKBIE-deleted patients. In addition, as constitutive NF-κB activation has been observed in most, if not all, CLL patients, this may also override potential relevant yet subtler differences in gene expression between NFKBIE-deleted and WT patients.

Altered response to ibrutinib in NFKBIE-deleted cases

Finally, because truncating IkBε mutations appeared to lead to constitutive NF-κB activation independent of BcR signaling, we hypothesized that for NFKBIE-deleted patients no difference should be observed in the tumor cell response to ibrutinib (n = 3) and WT (n = 2) samples, which revealed an increase in the nuclear fraction of p50 and p65 in NFKBIE-deleted patients (Fig. 5, A–D). Hence, loss of IkBε inhibitory function increased nuclear p50/p65 translocation and consequent NF-κB activation.

NFKBIE deletion has limited impact on the global gene expression profile

To further investigate the impact of this truncating mutation, using shRNA, we knocked down the expression of IkBε (by 56% and 60%, in two independent experiments) in the HG3 CLL cell line (Rosén et al., 2012), which revealed differential gene expression profiles between the knockdowns and the parental as well as the mock-transfected cell line (Fig. 5, E and F; and Table S12). Gene annotation enrichment analysis using the DAVID Bioinformatics Resources revealed that the top annotation clusters included regulation of apoptosis and cell death and regulation of the NF-κB signaling pathway (Table S13). Because the HG3 cell line is EBV transformed, which may seriously interfere with BcR signaling (Siemer et al., 2008), we next studied the global gene expression patterns in primary CLL cells from nine NFKBIE-deleted (mean allele frequency 45%, range 28–61%) and nine WT patients. Although an interesting up-regulation of several small nuclear RNAs (i.e., SNORD66, SNORD114-1, and SNORA80-B), previously linked to cancer (Gao et al., 2015), was observed in the NFKBIE-deleted group, only a few genes were significantly differentially expressed between the subgroups (Table S14). This finding might reflect previous gene expression profiling studies in U-CLL and M-CLL, showing only subtle differences in gene expression signatures in these clinically distinct subgroups (Klein et al., 2001; Rosenwald et al., 2001); along this line, one gene known to be up-regulated in U-CLL is ZAP70, and this gene also showed a higher expression in NFKBIE-deleted patients. In addition, as constitutive NF-κB activation has been observed in most, if not all, CLL patients, this may also override potential relevant yet subtler differences in gene expression between NFKBIE-deleted and WT patients.

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Finally, because truncating IkBε mutations appeared to lead to constitutive NF-κB activation independent of BcR signaling, we hypothesized that for NFKBIE-deleted patients no difference should be observed in the tumor cell response
the BcR inhibitor ibrutinib after αIgM stimulation. To test this hypothesis, we treated primary CLL cells from four NFKBIE-deleted and four NFKBIE WT patients with ibrutinib, in the presence or the absence of αIgM stimulation. A difference in cell survival was observed between unstimulated versus stimulated IκBα-WT cells, whereas no such difference was seen in IκBα-mutated cases (Fig. 5 G), which were generally more sensitive to ibrutinib than WT patients. Although at first sight the finding that NFKBIE-deleted cases were generally more sensitive to ibrutinib than WT patients may seem counterintuitive, a similar observation has been reported for the ABC type of DLBCL and has been attributed to tonic activation of the BcR–NF-κB signaling pathway. Thus, along the same lines, one could reasonably hypothesize that because of tonic BcR signaling, NFKBIE mutant CLL cases could be more dependent on external stimulation and, hence, more sensitive to BTK inhibition (Davis et al., 2010; Mathews Griner et al., 2014).

In summary, we provide for the first time a novel genetic basis for NF-κB activation with the prime finding being recurrent mutations in genes belonging to the NF-κB pathway and in particular within the NFKBIE gene not only in CLL but also in other B cell–derived malignancies. In CLL, we show that NFKBIE aberrations were highly enriched in poor-prognostic, stereotyped subsets, potentially contributing to their adverse prognosis, and resulted in reduced IκBα
protein levels and diminished interactions between IkBα–p65, as well as increased p65 phosphorylation and nuclear translocation. Notably, minor clones and/or clonal evolution were also observed, thus potentially linking IkBα loss to disease progression. Considering the central role of BcR stimulation in the natural history of CLL, the functional loss of IkBα may significantly contribute to sustained CLL cell survival in these patients. On these grounds, components of the NF-κB signaling pathway may emerge as possible targets for future therapies in CLL and, possibly, also other mature B cell lymphomas.

MATERIALS AND METHODS

Patient samples. In total, 692 CLL samples were collected from collaborating institutions in Sweden, Greece, Italy, France, Czech Republic, the Netherlands, the USA, and the UK. All cases were diagnosed according to the iwCLL criteria, displayed a typical CLL immunophenotype, and contained >70% tumor cells (Halleck et al., 2008). Clinicobiological characteristics of the discovery and validation cohorts are summarized in Tables S2 and S5. Mantle cell lymphoma (n = 136), DLBCL (n = 66), and splenic marginal zone lymphoma (n = 170) samples were diagnosed according to the WHO classification. The study was approved by the local Ethics Review Committees (Bromo: NT13493-4/2012, Milan: VIVI-CLL, New York: 08-202A, Thesaloniki: CERTH/ETH2, Uppsala: 214/33, Southampton: 06/Q2202/30, and Stockholm: 2006/964-31/2).

Targeted enrichment and library construction. We applied HaloPlex technology (Agilent Technologies) for targeted enrichment. For the discovery cohort, an earlier version of the current HaloPlex protocol was used. Biotin-labeled probes were designed for 18 NF-κB genes that target all coding exons with a high coverage (Tables S1 and S6). In brief, genomic DNA was fragmented using a combination of restriction enzymes. Biotin-labeled HaloPlex probes were hybridized to the target DNA and acted as template for a second universal DNA oligonucleotide, which contains primer sites, sequencing barcodes, and adapter sequences. The target DNA and the hybridized molecules were captured using streptavidin-labeled magnetic beads and circularized after a ligation reaction. The circularized DNA was amplified using universal primers. For the validation cohort, we took advantage of the automated HaloPlex protocol (http://www.chem.agilent.com/Library/usermanuals/Public/G9900-90020.pdf) using a Bravo Automated Liquid Handling Platform (Agilent Technologies). The libraries were subsequently sequenced using a HiSeq 2000 sequencing system (Illumina).

Targeted sequencing data analysis. Illumina adapters were trimmed using Cutadapt, and the reads were aligned to the human genome reference hg19/NCBI GRCh37 using the MOSAIK alignment tool version 2.2 (Martin, 2011). SAMTools was used for file format conversion and sorting. Using a modified version of GATK-lite, the aligned reads were mapped to their corresponding HaloPlex fragment. Variants were detected using an in-house, purpose-built variant caller (SNPmania) and annotated using ANNOVAR (Wang et al., 2010). Exonic variants were kept if they fulfilled the following criteria: (a) having a variant allele ratio of ≥0.1, (b) not in dbSNP and/or 1,000 Genomes or annotated in the ExAC database, and (c) supported by at least two amplicons unless the position was covered by a single amplicon only.

Sanger sequencing and GeneScan analysis. Selected variants were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit and an ABI 3730 DNA Analyzer (Life Technologies) using standard protocols. For GeneScan analysis, the following oligonucleotides were used for the analysis of the 4-bp deletion in NFKBIE: forward primer, 5′-Hex[CCTCAAAAGTGGGCTGAG-3′; reverse primer, 5′-CAAGGACACCAGGAGG-3′. Genomic DNA was amplified by hot-start PCR with Platinum-Taq DNA Polymerase (Invitrogen) and 60°C as annealing temperature. The fragment length of the PCR products was assessed by capillary electrophoresis with ABI3730XL DNA Analyzer (Applied Biosystems) and analyzed with Peak Scanner Software v1.0 (Applied Biosystems).

Western blots, co-IP assays, and cell fractions. Primary CLL cells were washed in PBS and lysed for 10 min in ice-cold RIPA buffer supplemented with phosphatase/protease inhibitors (Roche). Crude cell lysates were cleared by centrifugation and supernatants were transferred to new tubes. For collection of total cell lysates (TCLs), the supernatants were immediately mixed with 4× NuPAGE LDS sample buffer (Life Technologies) with DTT and treated at 95°C for 5 min. For co-IP assays, supernatants were first incubated 2 h at 4°C (end-over-end rotation) with 1:100 addition of either an anti-p65 antibody (a detailed list of all antibodies used is provided in Table S15) or a non-p65–targeting control antibody (AMLS) followed by addition of protein A agarose beads (Cell Signaling Technology) and an extra hour of incubation. Beads were collected by centrifugation and washed three times in ice-cold RIPA buffer before being diluted in NuPAGE LDS Sample buffer (Life Technologies) and transferred to nitrocellulose membranes using iBlot (Life Technologies). Blocking was performed at room temperature by incubating membranes for 1 h in 5% wt/vol nonfat dry milk (Bio-Rad Laboratories) or 5% wt/vol BSA (Sigma-Aldrich) in TBS buffer. Primary antibodies were diluted in TBS-T (0.1% Tween-20) with 5% wt/vol nonfat dry milk (Bio-Rad Laboratories) or 5% wt/vol BSA (Sigma-Aldrich) and were incubated together with the blocked membranes at 4°C overnight. Before imaging, membranes were washed (3× 20 min) in TBS-T (0.1% Tween-20), incubated 1 h with secondary antibodies (1:10,000/1:20,000 of the IRDye 800CW goat anti-rabbit IgG and/or 1:20,000 of the IRDye 680RD donkey anti–mouse IgG [LI-COR Biosciences]) in TBS-T (0.1% Tween-20) with 5% wt/vol BSA, washed (3× 15 min) in TBS-T (0.1% Tween-20), and finally rinsed 5 min in TBS buffer only. All incubation steps were performed at room temperature. Membranes were scanned and imaged using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences). Protein bands were quantified using ImageJ software (National Institutes of Health).

Stimulation of BcR and CD40 signaling pathways. Two million viable primary CLL cells per milliliter were grown in RPMI medium supplemented with 10% FBS ( Gibco), 1% PEST (Gibco), and 1% L-glutamine (Gibco), supplemented with 25% vol/vol serum-free supernatant from the T cell hybridoma cell line MP6 (Rosén et al., 1986), as a source of thioredoxin (Soderberg et al., 1999; Nilsson et al., 2000). For stimulation of the BcR, the modified RPMI medium was supplemented with 3 μg/ml AffiniPure F(ab′)2 fragment rabbit anti-human IgM (Jackson ImmunoResearch Laboratories, Inc.), 10 ng/ml IL-2 (GE Healthcare), and 1 μg/ml streptavidin (Roche). To stimulate the CD40 signaling pathway, cells were treated with the modified RPMI medium supplemented with 100 ng/ml sCD40L (Enzo Life Sciences), 25 ng/ml IL-4 (R&D Systems), and 100 ng/ml IL-10 (R&D Systems). Cells were stimulated for 15 min at 37°C (5% CO2). Cytospins were prepared using 150,000 cells per slide and a Celspin 1 cytospin centrifuge (Tharc) at 500 rpm for 2 min.

Proximity ligation assay. The cells were fixed in 3.7% formaldehyde solution (Sigma-Aldrich) for 15 min at room temperature and permeabilized in 0.5% Triton X-100 (GE Healthcare) for 2 min at room temperature. All washing steps were performed twice for 5 min in 1× TBS with 0.05% Tween (Sigma-Aldrich) unless stated otherwise. First, the samples were incubated in a blocking solution (Olink Biosciences) for 1 h at 37°C. The primary antibodies were dilute as follows, p65 (Cell Signaling Technology) 1:400, 1×60 (Cell Signaling Technology) 1:50, 1×60B (Santa Cruz Biotechnology, Inc.) 1:50, 1×60E (Santa Cruz Biotechnology, Inc.) 1:200, and 1×60B (Cell Signaling Technology) 1:50 in antibody diluent solution (Olink Biosciences) and applied to the samples for incubation overnight at 4°C. The samples were then washed and incubated with Duolink In Situ PLA Probe

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anti–mouse MINUS Affinity donkey anti–mouse IgG and Duolink In Situ PLA Probe anti–rabbit PLUS Affinity donkey anti–rabbit IgG (Olink Bioscience) diluted 1:5 in antibody diluent solution (Olink Biosciences) for 1 h at 37°C, followed by washing. A hybridization solution containing 0.25 mg/ml BSA (New England Biolabs, Inc.), 25 mM NaCl, 0.05% Tween-20 (Sigma-Aldrich), 10 mM TrisAc, 10 mM MgAc, 50 mM KAc, and 125 mM of circularization oligonucleotides (5′-GTTCGTCTATATGGTAAATTTGGTCTATGTTAAGATCGGTCCGT-CTAAAGGAGTTAGTCAGCCGCTGACTGCGAG-3′). Integrated DNA Technology at pH 7.5 were applied to the sample for 30 min incubation at 37°C, followed by washing. Both oligonucleotides were phosphorylated in the 5′ end. Ligation of the circularization oligonucleotides were performed in 1× T4 ligation buffer (Fermentas, Thermo Fisher Scientific), 0.05 U/μl T4 DNA ligase (Fermentas), and ddH2O for 30 min at 37°C, followed by washing. Rolling circle amplification was performed by incubating the samples in 0.25 mg/ml BSA (New England Biolabs, Inc.), 300 ng/ml poly-adenosine, 1× phi29 DNA polymerase buffer (Fermentas), 0.25 mM dNTP (Thermo Fisher Scientific), 1 μM Hoechst 33342 (Sigma-Aldrich), 0.25 U/μl phi29 DNA polymerase (Fermentas), and a BODIPY TR–labeled oligonucleotide (5′-CAGTGATGTCGCTCCGTTCUULU3′, U represents Uracil 2′O methyl RNA group, Trilink) for 90 min at 37°C. Finally, the samples were washed twice in 1× TBS supplemented with Tween (Sigma-Aldrich) and once in 1× TBS, followed by centrifugation of the slides. SlowFade Gold antifade reagent (Life Technologies) was used for mounting of the slides. Images were acquired with an Axioplan 2 imaging microscope (Carl Zeiss) using 4× objectives and an AxioCam MRm camera (Carl Zeiss). Exposure times, number of z-levels, and distance between z-levels were kept the same for all patients within each assay. CellProfiler version one was used to quantify number of signals per cell in raw images. The interactions between the iKDs and p65 were normalized against the number of interactions between p65 and p50 for each sample.

Stable knockdown of iKBe. Stable knockdown of iKBe in the HG3 CLL cell line was established using the pGIPZ lentiviral vector (V3LHS_365665) and the mature antisense sequence 5′-TGGTCCAGATGTACAGCCA-3′ (GE Healthcare). The plasmid was linearized using SpeI restriction enzyme (Fermentas). HG3 cells were transfected with 3 μg of plasmid DNA per 10⁷ cells by electroporation using the Neon Transfection System (Thermo Fisher Scientific) and standard parameters. Puromycin selection was started 48 h after transfection and cells were kept under Puromycin selection. Additional selection for positive clones was performed by FACS sorting for cells expressing tGFP, expressed from the same promoter as the shRNA and the Puromycin resistance gene.

Gene expression analysis. Gene expression was studied using Affymetrix GeneChip Human Gene 2.0 ST Arrays (Affymetrix) and 250 ng total RNA according to standard protocols. Data were analyzed in R. (The R Project for Statistical Computing) using packages from the Bioconductor project and normalized using the robust multi-array average method (Irizarry et al., 2003). To search for differentially expressed genes, an empirical Bayes–moderated Student’s t test was applied using the “limma” package. The p-values were adjusted using the method of Benjamini and Hochberg to address potential problems with multiple testing (Benjamini and Hochberg, 1995). Genes with an adjusted p-value <0.05 were regarded as differentially expressed. Gene annotation enrichment analysis was performed using the DAVID Bioinformatics Resources.

Ibrutinib treatment and cell viability test. Unstimulated and algM-stimulated (10 μg/ml for 15 min) primary CLL cells from four NFκBIE WT patients and four NFκBIE-deleted patients were plated in quadruplicate wells at a density of 100,000 cells per well in 96-well plates followed by ibrutinib (PCI-32765; Selleckchem) treatment at 0, 0.1, 1, 2.5, and 10 μM concentration for 72 h. Alamar Blue (Life Technologies) was added, and cell viability was measured after 24 h using a VICTOR plate reader (PerkinElmer) and standard protocols. Data were normalized against ibrutinib naive matched controls.

Statistical analysis. Paired Student’s t test was used to assess differences between subgroups with at least four patients in each subgroup. Friedman ANOVA was used to study differences in NFκBIE mutation frequency among CLL subset cases. Kaplan–Meier analysis was performed to construct survival curves for TTFT, defined as the time interval from the diagnosis date until date of initial treatment, and the log-rank test was used to assess differences. All statistical analyses were performed using statistical version 12 (Stat Soft).

Online supplemental material. Table S1 shows the 18 NF-κB core complex genes targeted for deep sequencing in the discovery cohort. Table S2 shows the clinical and biological characteristics of CLL patients in the discovery cohort. Table S3 shows a summary of mutations found in the discovery cohort (n = 124) with additional molecular data. Table S4 shows low-frequency NFκBIE deletions (<10%) detected in the discovery (n = 124) and validation cohorts (n = 191). Table S5 shows clinical and biological characteristics of CLL patients in the validation cohort. Table S6 shows the 18 NF-κB core complex genes targeted for deep sequencing in the validation cohort. Table S7 shows a summary of mutations found in the validation cohort (n = 191). Table S8 shows clinical and biological characteristics of the population-based CLL cohort. Table S9 shows a summary of NFκBIE deletions detected by GeneScan analysis (n = 383). Table S10 shows multivariate analyses for TTFT (A) and for TTFT excluding IGHV mutation status (B). Table S11 shows allele ratio of the deleted NFκBIE allele detected by GeneScan analysis. Table S12 shows differentially expressed genes in the HG3 cell line after NFκBIE knockdown. Table S13 shows gene annotation enrichment analysis performed using the DAVID Bioinformatics Resources for differentially expressed genes in HG3 cell line after NFκBIE knockdown. Table S14 shows a list of significantly differentially expressed genes in NFκBIE-deleted versus NFκBIE WT cases. Table S15 shows the list of antibodies used. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20142009/DC1.

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