

Deregulation of the endogenous C/EBP β LIP isoform predisposes to tumorigenesis

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Abstract

Two long and one truncated isoforms (termed LAP*, LAP, and LIP, respectively) of the transcription factor CCAAT enhancer binding protein beta (C/EBP β) are expressed from a single intronless *Cebpb* gene by alternative translation initiation. Isoform expression is sensitive to mammalian target of rapamycin (mTOR)-mediated activation of the translation initiation machinery and relayed through an upstream open reading frame (uORF) on the C/EBP β

mRNA. The truncated C/EBP β LIP, initiated by high mTOR activity, has been implied in neoplasia, but it was never shown whether endogenous C/EBP β LIP may function as an oncogene. In this study, we examined spontaneous tumor formation in C/EBP β knockin mice that constitutively express only the C/EBP β LIP isoform from its own locus. Our data show that deregulated C/EBP β LIP predisposes to oncogenesis in many tissues. Gene expression profiling suggests that C/EBP β LIP supports a pro-tumorigenic microenvironment, resistance to apoptosis, and alteration of cytokine/chemokine expression. The results imply that enhanced translation reinitiation of C/EBP β LIP promotes tumorigenesis. Accordingly, pharmacological restriction of mTOR function might be a therapeutic option in tumorigenesis that involves enhanced expression of the truncated C/EBP β LIP isoform.

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Key message

- Elevated C/EBP β LIP promotes cancer in mice.
- C/EBP β LIP is upregulated in B-NHL.
- Deregulated C/EBP β LIP alters apoptosis and cytokine/chemokine networks.
- Deregulated C/EBP β LIP may support a pro-tumorigenic microenvironment.

Keywords Tumorigenesis · Lymphomagenesis · Translational control · Tumor-stroma interaction

Introduction

The CCAAT enhancer binding protein beta (C/EBP β) transcription factor belongs to the basic leucine zipper (bZip) C/

EBP family and controls cell fate in many tissues. C/EBP β is involved in cell growth, proliferation, differentiation, apoptosis, senescence, and tumor tolerance. *Cebpb* is a single exon gene, yet three isoform proteins of variable N-terminal length are expressed from internal AUG start sites. The two long isoforms (C/EBP β liver-enriched activator proteins LAP* and LAP) are both transcriptional activators, but differ by 21 N-terminal amino acid residues that entail chromatin regulatory capacity. The truncated C/EBP β liver inhibitory protein (LIP) isoform lacks the N-terminal 185 amino acid residues, removing the entire transactivation domain and part of the regulatory domain. C/EBP β LIP is thought to dominantly counteract tumor suppressive functions of other C/EBP family members, suggesting C/EBP β LIP as a potential oncogene [1, 2].

A highly conserved (from fish to man) small upstream open reading frame (uORF) in the 5' mRNA region regulates alternative translation initiation of *Cebpb* at consecutive in frame start sites. Previous data showed that translation of the uORF restrains initiation of C/EBP β LAP and causes resumption of ribosomal scanning and reinitiation at the downstream C/EBP β LIP start site. The translational control and C/EBP β isoform switching depends on mammalian target of rapamycin (mTOR) signaling [3–6], stress response pathways [7], and RNA-binding proteins [8]. Briefly, at high activity of the translation initiation machinery the C/EBP β LIP isoform is preferentially produced, whereas at low activity, the long C/EBP β LAP isoform is preferentially produced [9, 10]. Recent evidences suggested that the C/EBP β LAP/LIP ratio is an important indicator of C/EBP β functions. Regulation of C/EBP β LAP/LIP ratio plays a key role in liver regeneration, acute phase response, bone homeostasis, and mammary gland development [9, 10]. Enhanced expression of C/EBP β was observed in human tumors, including mammary carcinomas [11–13], anaplastic large cell lymphoma (ALCL) [14–16], endometrial adenocarcinoma [17], ovarian [18], colorectal [19], liver [7], and skin cancer [20]. Among these, enhanced expression of the truncated C/EBP β LIP isoform was reported in breast, ALCL, ovarian, and colorectal neoplasia. C/EBP β LIP also supports metastasis survival in breast cancer [21] and pharmacological inhibition of C/EBP β LIP expression reduced ALCL proliferation, implying a causal relationship between C/EBP β LIP isoform expression and tumorigenesis [14].

Here, we used recombinant mouse genetics to explore whether deregulated expression of C/EBP β LIP from its own genomic locus supports spontaneous tumor formation. A knockin mouse strain that expresses the C/EBP β LIP isoform (LIP ki) developed tumors in multiple tissues of mesenchymal and epithelial origin, providing experimental evidence that enhanced translation reinitiation of C/EBP β LIP is involved in tumorigenesis.

Materials and methods

Animals

The generation of *Cebpb* knockout (ko) and LIP ki mice have been previously described [9, 22]. The C/EBP β ko and LIP ki mice were maintained on a 129 \times C57BL/6 genetic background. Mice were fed with standard mouse diet and water ad libitum on a 12-h light-dark cycle. Animals were housed in a pathogen-free facility at the Max Delbrück Center for Molecular Medicine, Berlin. All procedures and animals experiments were conducted in compliance with protocols approved by the institutional Animal Care and Use Committee. Mice were sacrificed by cervical dislocation. All efforts were made to minimize animal suffering.

Histopathological analyses

Mice were sacrificed upon substantial decline in health (i.e., weight loss, paralysis, ruffling of fur, or inactivity) or obvious tumor burden. Tissues were fixed in buffered 4 % formaldehyde at 4 °C, dehydrated, and paraffin-embedded. Sections (4 μ m) were stained with hematoxylin and eosin according to standard procedures. To perform immunohistochemical (IHC) staining slides were immersed in sodium citrate buffer solutions at pH 6.0, heated in a high-pressure cooker for 5 min, rinsed in cold water, and washed in Tris-buffered saline (pH 7.4), and were then treated with a peroxidase-blocking reagent (Dako) before incubation for 1 h with the primary antibody for B220 (RA3-6B2, eBioscience) or cleaved caspase-3 (Asp175, Cell Signaling). Binding was detected by the Envision peroxidase kit (K4010, Dako) using diaminobenzidine as chromogenic substrate or by the streptavidin alkaline phosphatase kit (K5005, Dako). Sections were analyzed using an AxioPlan-2 microscope (Zeiss, Germany). Images were acquired using a Zeiss AxioCam Hr camera and AxioVision software version 4.2. For cleaved caspase-3 IHC analysis, per animal, five randomly chosen microscope fields were captured at \times 200 magnification. The number of cleaved caspase-3-positive cells/field were counted and expressed as fold of control.

Cell culture and immunoblotting

Mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos and spontaneously immortalized according to standard protocols. 3T3-L1 murine preadipocytes (American Type Culture Collection (ATCC)) and MEFs were cultured in DMEM (Invitrogen) supplemented with 10 % FBS (Invitrogen). Tissues, lymphomas, and cells were lysed with 8 M urea lysis buffer. Proteins were separated by SDS-PAGE, followed by Western blot analysis using rabbit C/EBP β antibody (C19, Santa Cruz) and mouse anti- α -tubulin (B-7, Santa Cruz) or mouse anti- β -tubulin (2-28-33, Sigma). Appropriate

horseradish peroxidase-conjugated secondary antibodies were used for chemiluminescence (Amersham Biosciences).

RNA preparation and microarray gene expression profiling

Total RNA from 5 *C/EBPβ* LIP heterozygous (+/L) and 5 wild-type (WT) lymphomas was prepared using the TriPure Isolation Reagent (Roche) and analyzed on a 4×44K whole genome mouse microarray (Agilent Technologies). For normalization, quality control, and data analysis, the Bioconductor system was used (<http://www.bioconductor.org>) [23]. Raw microarray data from Agilent were first quantile normalized. For identification of differentially expressed genes, a linear model was fitted using limma. The fold change cutoff was set to 2 (FC=2), and the *P* value cutoff was selected as 0.05. For functional analysis, gene set enrichment analysis (GSEA) was performed using GSEA v2.0 algorithm (<http://www.broadinstitute.org/gsea>) [24] and the computed *t*-statistic from limma as pre-ranking. The following gene sets from MSigDB database (<http://www.broadinstitute.org/gsea>) were used: C2: curated sets, canonical pathways, Biocarta, KEGG, Reactome and C5: Go gene sets, BP: biological process. Only gene sets showing nominal *P* value ≤0.05 and false discovery rate (FDR) ≤0.25 were taken into consideration.

Statistical analysis

All data are expressed as mean±SEM. Statistically significant differences between groups were determined using the unpaired two-tailed Mann-Whitney's test for the histograms and the log-rank test for the Kaplan-Meier curves. Both tests were performed using Prism 5 (GraphPad Software). A *P* value <0.05 was considered to be statistically significant.

Accession number

The raw microarray data were deposited at Gene Expression Omnibus (GEO accession number GSE53770).

Results

LIP ki mice are cancer prone

An excess of *C/EBPβ* LIP has been suggested to promote metastatic breast cancer by interfering with the TGFβ cytosolic pathway and/or by inhibition of anoikis [21, 25]. Moreover, expression of *Cebpb* LIP as a transgene under the whey acidic promoter led to mammary gland hyperplasia and rare neoplasia [26]. Although these experimental approaches have hinted at an oncogenic potential of *C/EBPβ* LIP, they did not

reflect organismal constraints in quantitative, spatial, and temporal *Cebpb* regulation from its own locus.

To examine whether enhanced endogenous *C/EBPβ* LIP expression is involved in tumorigenesis, cohorts of 45 wild-type (+/+, WT), 52 *C/EBPβ* LIP homozygous (L/L), and 72 *C/EBPβ* LIP heterozygous (+/L) mice were monitored over a period of more than 25 months. Protein analysis confirmed expression of *C/EBPβ* LIP in the same tissues as *C/EBPβ* in WT mice (Fig. 1a). Figure 1b illustrates the survival of +/L and L/L mice as compared to their WT siblings. Median survival for WT mice was 24 months, versus 20 months for +/L, and 17 months for L/L mice. These results showed that deregulated expression of the *C/EBPβ* LIP isoform decreased survival in a dominant and dose-dependent manner.

At 20 months of age, a 3.5-fold increase in tumorigenesis was observed in L/L mice as compared to WT littermates (Table 1 and Fig. 1c). Histopathological analyses of the solid tumors of L/L mice revealed B cell non-Hodgkin's lymphoma (B-NHL, 25 % in L/L vs 9 % in WT), histiocytic sarcoma (HS, 5.8 % in L/L vs 0 % in WT), and lung adenocarcinoma (1.9 % L/L vs 0 % in WT) (Table 1 and Fig. S1).tgroup

Mice that express one WT *Cebpb* allele and *Cebpb* LIP from the second allele (+/L) reflect translational deregulated, enhanced endogenous *C/EBPβ* LIP expression. At 20 months of age, 37.5 % of the +/L mice had developed solid tumors, in comparison to 9 % of the WT mice (Table 1 and Fig. 1c). Compared to L/L mice, the incidence of B-NHL was slightly increased in +/L (32 % in +/L vs 25 % in L/L), yet the incidence of histiocytic sarcoma and lung adenocarcinoma remained similar (histiocytic sarcoma, 7 % in +/L vs 5.8 % in L/L; lung adenocarcinoma, 1.4 % in +/L vs 1.9 % in L/L) (Table 1). A cohort of *Cebpb* heterozygous (*Cebpb*^{+/-}) mice were kept under the same conditions. Median survival was similar in both *Cebpb*^{+/-} and WT littermates (cohort, 25 *Cebpb*^{+/+} and 27 *Cebpb*^{+/-} mice; median survival, 24 and 25 months, respectively) and no tumor was found at 20 months of age (data not shown). Investigation of spontaneous tumor formation in *Cebpb*^{-/-} mice was not possible due to occurrence of infection and severe skin wound phenotype before 1 year of age in more than 60 % of the mice. Altogether our data suggest that increase in *C/EBPβ* LIP is responsible for tumor development (as in +/L mice) but not a decrease in total *C/EBPβ* (as in *Cebpb*^{+/-} mice).

B-NHL was found at high incidence in the LIP ki mice. As shown in Fig. 1d, LIP ki mice developed B-NHL significantly earlier than WT, although the 129 × C57BL/6 strain is known to be susceptible to B-NHL formation at old age [27]. Interestingly, expression of *C/EBPβ*, and in particular of the *C/EBPβ* LIP isoform, is high in lymphomas that develop in WT mice (Fig. 1e), in comparison to the spleens of young WT or LIP ki mice (Fig. 1a) and to the isolated CD19⁺ B cells (data not shown). Moreover, the tumor spectrum in +/L mice was broader than that in L/L

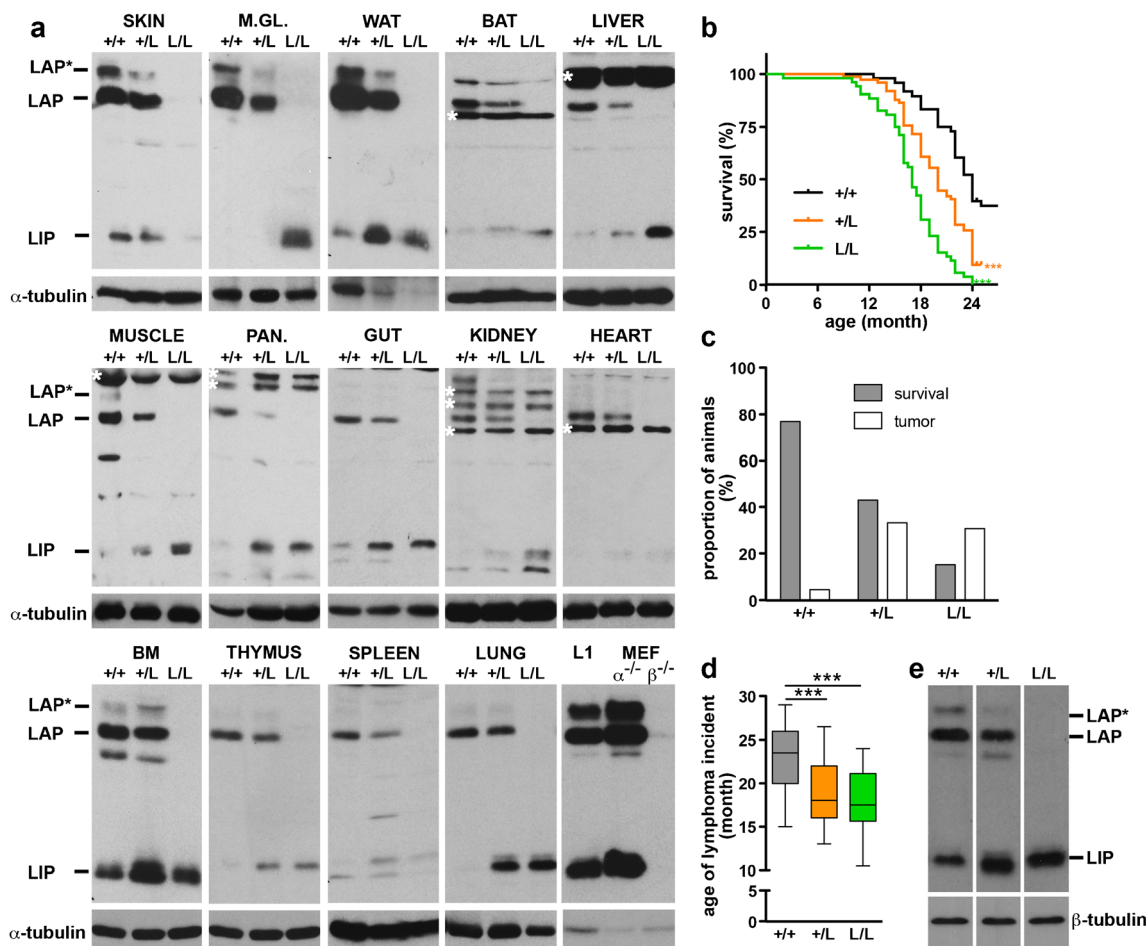


Fig. 1 LIP ki mice are cancer prone. **a** C/EBP β isoform expression in tissues of WT and LIP ki mice. Various tissues isolated from 8-week-old WT and LIP ki mice (+/L, L/L) were lysed in 8 M urea lysis buffer and analyzed by Western blotting for expression of C/EBP β isoforms LAP*, LAP, and LIP (as indicated). *M.GL.* mammary gland, *WAT* white adipose tissue, *BAT* brown adipose tissue, *Pan* pancreas, *BM* bone marrow. 3T3-L1 cells (L1) and *Cebpa*^{-/-} MEF ($\alpha^{-/-}$) were used as positive controls whereas *Cebpb*^{-/-} MEF ($\beta^{-/-}$) were used as negative controls. α -Tubulin was used as internal control. Unspecific immune reactivity (*white asterisk*). **b** Dosage effect of C/EBP β LIP on survival rate. Kaplan-Meier curve of +/+ (black line) and LIP ki mice (+/L, orange line; L/L, green line) housed over a period of 25 months. Mice were monitored twice weekly for tumor formation. Moribund mice or mice showing fatal illness or tumor development were sacrificed, and tissues were isolated for further examination. Cohorts of mice: +/+, $n=45$; +/L, $n=72$; L/L, $n=$

52. Accelerated death of +/L and L/L mice versus +/+ mice and of +/L mice versus L/L mice according to the log-rank test was significant in each case: *** $P<0.0001$. **c** Comparison of survival rate and tumor incidence at 20 months of age between LIP ki mutants and WT mice: +/+ (4 tumors out of 45 +/+ mice); +/L (27 tumors out of 72 mice); L/L (16 tumors out of 52 mice). **d** Comparison of average age for lymphoma development in WT and LIP ki mice. Cohorts of mice: +/+, $n=45$; +/L, $n=72$; L/L, $n=52$. Error bars show SEM. Significant accelerated lymphoma development of +/L and L/L mice versus +/+ mice were analyzed using the unpaired two-tailed Mann-Whitney's test: *** $P<0.0001$. **e** Lymphomas found in WT and LIP ki mice were lysed in 8 M urea lysis buffer and analyzed by Western blotting for expression of C/EBP β isoforms LAP*, LAP, and LIP (as indicated). β -Tubulin was used as internal control

mice and included T-lymphoma, carcinoma of the skin, liver, and mammary gland (Table 1 and Figs. 2, S2, S3, and S4). Note that C/EBP β protein expression was detected in all tumor types (Figs. S1, S2, and S5). The generally low carcinoma incidence, however, may be masked by faster lymphoma development, as previously reported in other murine cancer models [28]. These data show that deregulated expression of C/EBP β LIP from its own locus enhances tumor development in several mesenchymal and epithelial tissues.

Deregulation of cancer pathways in LIP ki mice

To determine which signaling pathways were altered in LIP ki mice tumors, B-NHL tumors obtained from LIP ki and WT mice were examined for cytogenetic alterations using SKY analysis (Table S1). No obvious gross genomic or recurrent rearrangements correlated with enhanced C/EBP β LIP expression.

Next, gene expression profiling analysis revealed 123 genes as differentially expressed in lymphomas of +/L mice

Table 1 Tumor distribution in LIP ki mice at 20 months of age

Genotype	+/+	+/L	L/L
Number dead/cohort	11/45	41/72	44/52 ^a
Tumor incidence			
/cohort	4/45 (9 %)	27/72 (37.5 %) ^{***}	16/52 (30.8 %) ^{**}
/number dead	4/11 (36 %)	27/41 (66 %) [*]	16/44 (36 %)
B-NHL	4/45 (9 %)	23/72 (32 %)	13/52 (25 %)
T-lymphoma	0	1/72	0
Histiocytic sarcoma	0	5/72	3/52
Mammary gland carcinoma	0	2/72	0
HCC	0	1/72	0
Skin carcinoma	0	1/72	0
Lung tumor	0	1/72	1/52

Note that some mice developed multiple primary solid tumors. Tumor incidence reflects number of mice with any kind of tumor(s) even though an animal might develop more than one tumor

B-NHL B cell non-Hodgkin's lymphoma, *HCC* hepatocellular carcinoma

* $P < 0.02$; ** $P < 0.005$; *** $P < 0.0005$, unpaired *t* test

^aL/L mice show a high death rate but a lower tumor incidence in comparison to +/L mice. L/L mice developed more infection and abscess than +/L and +/+ mice

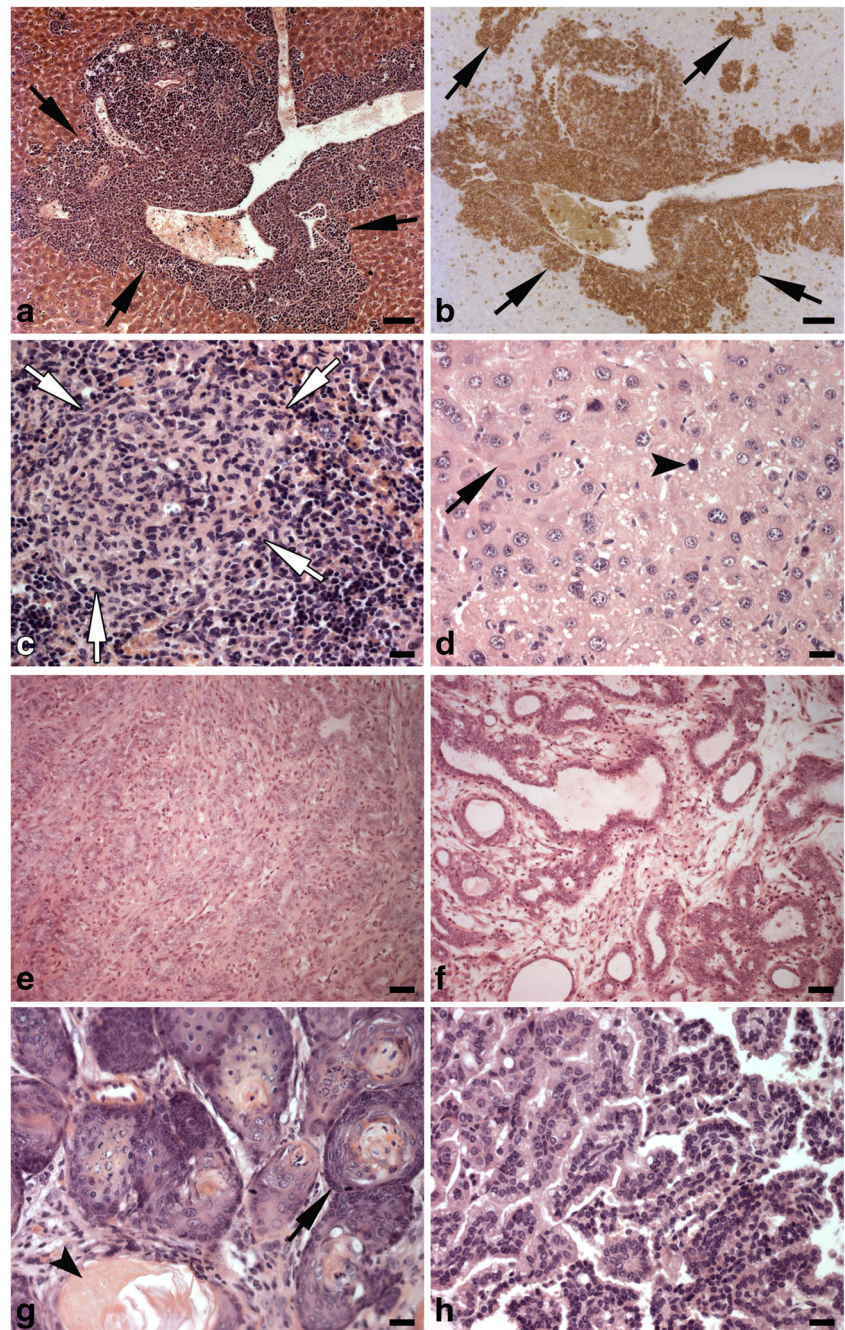
in comparison to lymphomas of WT mice (Fig. 3a and Table S2; 66 upregulated and 57 downregulated genes). GSEA and examination of leading-edge gene subsets identified enrichment of C2 and C5 functional sets in +/L mice that belong to mTOR pathways (<http://www.broadinstitute.org/gsea/msigdb>). In addition to mTOR signaling, gene sets involved in translation and regulation of translation, mitochondrial function, metabolism, IGF1, and FOXO pathways, are all significantly enriched in lymphomas of +/L mice (Fig. 3b–e and Table S3). These data support the notion that elevated C/EBP β LIP participates in metabolic signaling and mTOR regulated gene expression control during tumorigenesis.

In +/L lymphomas, GSEA highlighted three significant gene sets implicated in cell death signaling (Table S3). Moreover, the comparison between lymphomas of WT and +/L mice identified several gene sets involved in MAPkinase, ALK1, TGF β , and NF- κ B pathways that may affect apoptosis and that were significantly depleted in +/L lymphomas (Fig. S6 and Table S3). We also found that the number of apoptotic cells was slightly reduced in the spleen of +/L mice before tumor onset, whereas apoptosis rate was significantly increased in the spleen of *Cebpb*^{-/-} compared to WT mice (Fig. 4a). Furthermore, reduction of caspase-3 cleavage was detected in CD19⁺ B cells and lymphomas from +/L, as compared to WT (Fig. 4b, c, respectively). Similar results were obtained with spleen and CD19⁺ B cells from L/L mice (data not shown). Altogether, these data suggest that increase in C/EBP β LIP expression reduced the apoptotic rate in B cells and B-NHL.

Deregulation of immune defense might play a key role in tumorigenesis and several previous findings suggested C/EBP β as an important transcription factor controlling cytokine and chemokine expression in immune cells. Deregulated gene expression comprised known C/EBP β target genes, including *Saa3*, *S100a9*, *Arg1*, *Fpr1*, and *Cxcl13* (Table S2 [29, 30]). Gene expression profiling showed that approximately 14 % of the deregulated genes in +/L lymphomas encoded cytokines/chemokines (Fig. 5a and Table S2). Moreover, the comparison between lymphomas of WT and +/L mice using GSEA revealed leading-edge gene subsets involved in cytokine and chemokine biosynthesis, Toll-like receptor pathways, and innate immune response (Fig. 5b–d and Table S3). Expression levels of leukocyte recruiting Ccl3 and Ccl4 cytokines involved in tumor cell eradication, inflammatory M1 type classically activated macrophages (Cxcl13, Cxcl14, Cxcl16, Cx3cr1), and dendritic cells (Cxcl16, Cd11c) markers were all decreased in lymphomas of +/L mice (Fig. 5a). In contrast, M2-activated macrophage markers were enhanced in lymphomas of +/L mice (Fig. 5a; Cd36, Arg1, Ccl24, Mrc1, Retnla, Ccl11, Cd163). These data suggest association of a pro-tumorigenic microenvironment in LIP ki lymphomas.

Collectively, expression profiling and pathway analysis of C/EBP β LIP lymphomas revealed an increase in pro-tumorigenic cytokine release, deregulation of chemokine expression, and TLR signaling pathways, in addition to reduced apoptosis, that may all predispose and contribute to tumor susceptibility. This notion was further supported by recent evidence suggesting C/EBP β as a critical regulator of myeloid

Fig. 2 LIP *ki* mice develop mesenchymal and epithelial tumors. Histological analysis (H&E staining) of mesenchymal (**a, c**) and epithelial (**d–h**) tumors found in the *+L* mice. **a–b** B cell non-Hodgkin's lymphoma (B-NHL, **a**) with massive infiltration of B cells in the liver (*arrows* in **a**) characterized by B220 immunopositive staining (*arrows* in **b**) (see Fig. S1 for further characterization of the B-NHL). **c** A histiocytic sarcoma with nodular infiltrates (*arrows*) developed in the spleen (see Fig. S2 for further characterization of the histiocytic sarcoma). **d** Liver with a hepatocellular carcinoma (HCC) showing a trabecular growth pattern (*arrow*). *Arrowhead*: mitotic figure. **e–f** A ductal (**e**) and a tubular (**f**) mammary carcinoma developed in a *+L* female of 16 months of age. **g** Skin carcinoma with squamous differentiation and keratinization (*arrowhead*) as well as horn pearl formation (*arrow*). **h** Lung adenocarcinoma with papillary growth pattern. **a, b** Scale bar= 25 μ m; **c, d, g, h** scale bar= 20 μ m; **e, f** scale bar= 10 μ m (see Figs. S3 and S4 for comparison with nontumor tissues from age-matched *+/+* mice)

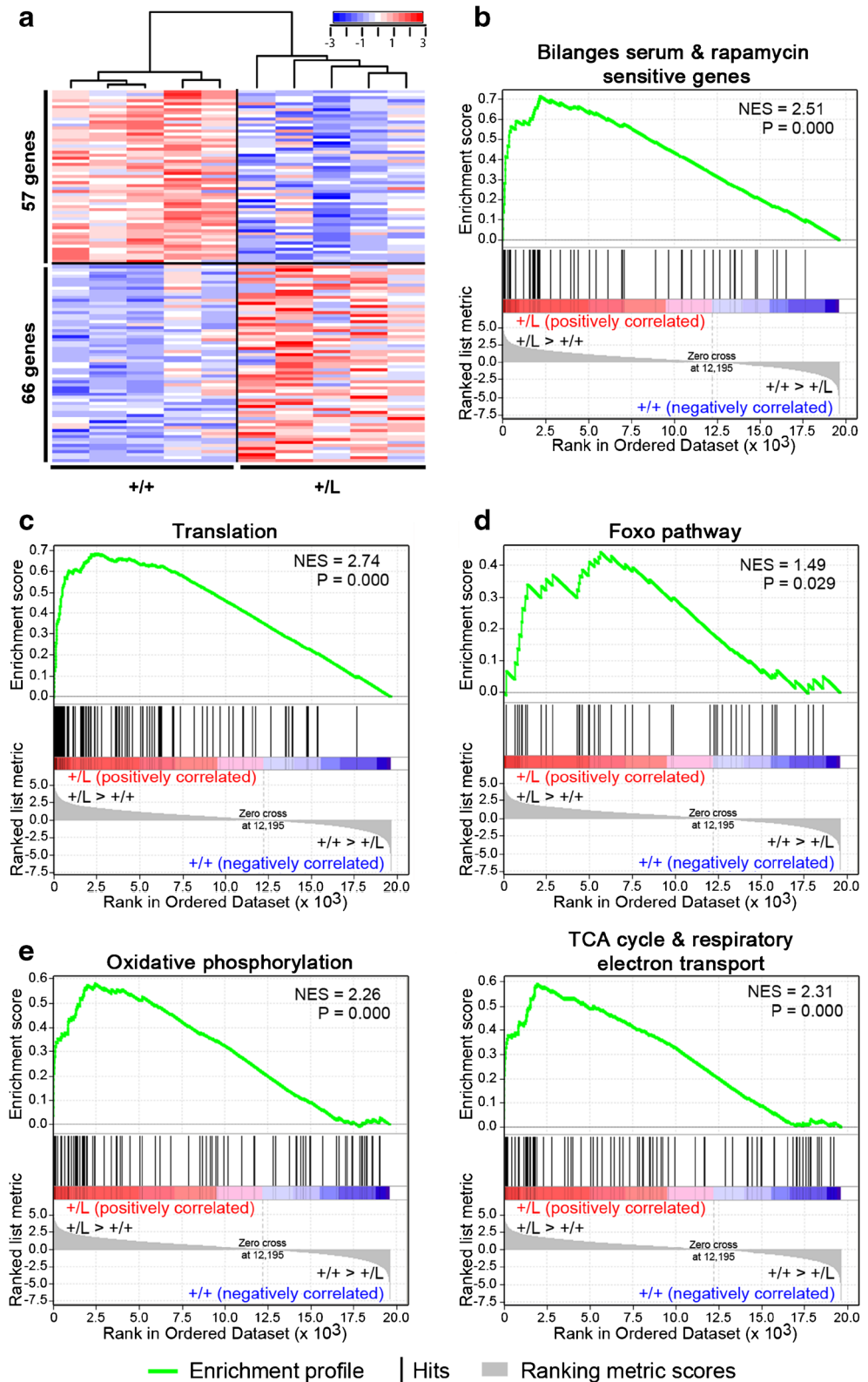


derived suppressor cells that promote the pro-tumorigenic immunosuppressive microenvironment [31].

To test whether deregulated *C/EBP β* LIP expression promotes lympho/myelo-proliferation, we performed bone marrow (BM) transfer of WT cells into lethally irradiated WT or *L/L* recipient mice (Fig. S7A). The distribution of hematopoietic cell lineages was different in WT BM reconstituted *L/L* and WT mice although engraftment of donor cells, and the

spleen weights were similar in both recipient strains (Fig. S7B and C). An increase in myeloid cells (CD11b-positive cells) and a decrease in T cells (CD3-positive cells) were found in the spleens of WT BM reconstituted *L/L* mice, in comparison to WT BM reconstituted WT mice (B220-positive cells were not affected; Fig. S7D). In peripheral blood, white blood cells (granulocytes, monocytes, lymphocytes) of WT donor origin were higher in *L/L* than in WT recipient mice (Fig. S7E and F),

Fig. 3 Gene expression profiling analysis of B-NHL of LIP ki mice. **a** Heat map of differentially regulated genes in B-NHL of +/L mice in comparison to B-NHL of WT mice as identified by gene array analysis. +/+, $n=5$; +/L, $n=5$ (see list of the genes in Table S2). **b–e** GSEA based on the comparison of B-NHL of +/L and WT mice for enrichment or depletion of rapamycin-sensitive genes (**b**), translation (**c**), FOXO pathway (**d**), and oxidative phosphorylation and TCA cycle and respiratory electron transport (**e**) associated genes. The normalized enrichment scores (NES) and P values are indicated in each plot. Note the positive NES values observed in all cases indicating an upregulation of these gene sets in B-NHL of +/L mice compared to B-NHL of WT mice



but no changes in red blood cell counts were observed (Fig. S7G). These data suggest that enhanced expression of the C/EBP β LIP isoform facilitates a tumor supportive

microenvironment, but further experiments are required to determine how the microenvironment is actually altered in LIP mice.

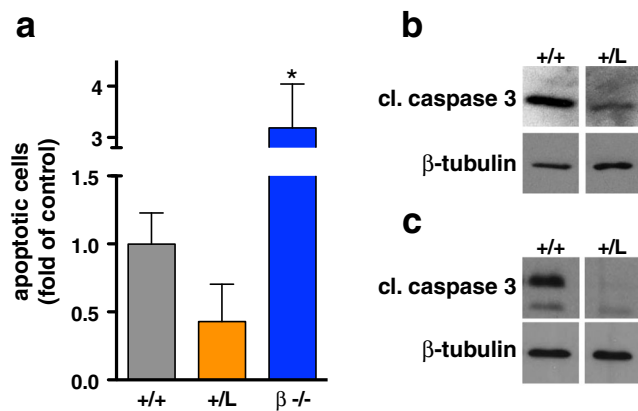


Fig. 4 Impaired apoptosis in LIP ki mice before and after tumor onset. **a** Number of apoptotic cells in the splenic white pulp of C/EBP β mutants and WT mice before tumor onset. Apoptotic cells were analyzed using cleaved caspase-3 immunostaining. Numbers of positive cleaved caspase-3 cells/field were counted and expressed as fold of control. $n=5$ per genotype. Error bars show SEM; * $P<0.05$. **b–c** Representative immunoblot analyses showing decrease in cleaved caspase-3 (cl.caspase 3) expression in CD19⁺ B cells sorted from spleen of 12-month-old LIP ki mice as compared with those of WT counterparts (**b**) and in B-NHL of LIP ki mice as compared with B-NHL of WT mice (**c**). β -Tubulin was used as loading control. The CD19⁺ B cells samples were run on the same gel but were noncontiguous (same for B-NHL samples)

Discussion

Data shown here firmly establish the proto-oncogenic function of endogenous C/EBP β LIP in mesenchymal and epithelial tissues. Tumors were found in tissues previously shown to depend on C/EBP β functions, including mammary gland, skin, liver, lung, and hematopoietic cells [15, 16, 30, 32–34]. Enhanced C/EBP β LIP expression leads to the development of follicular lymphoma (B-NHL) and histiocytic sarcoma. Human follicular lymphoma may eventually transdifferentiate into histiocytic sarcoma, and C/EBP β was found to be strongly expressed in these tumors [35]. C/EBP β LIP has also been shown to promote proliferation of human B cell Hodgkin's lymphoma and ALCL [14, 16], suggesting an important function of C/EBP β LIP deregulation in lymphomagenesis and hinting at similarities in disease development in rodents and human.

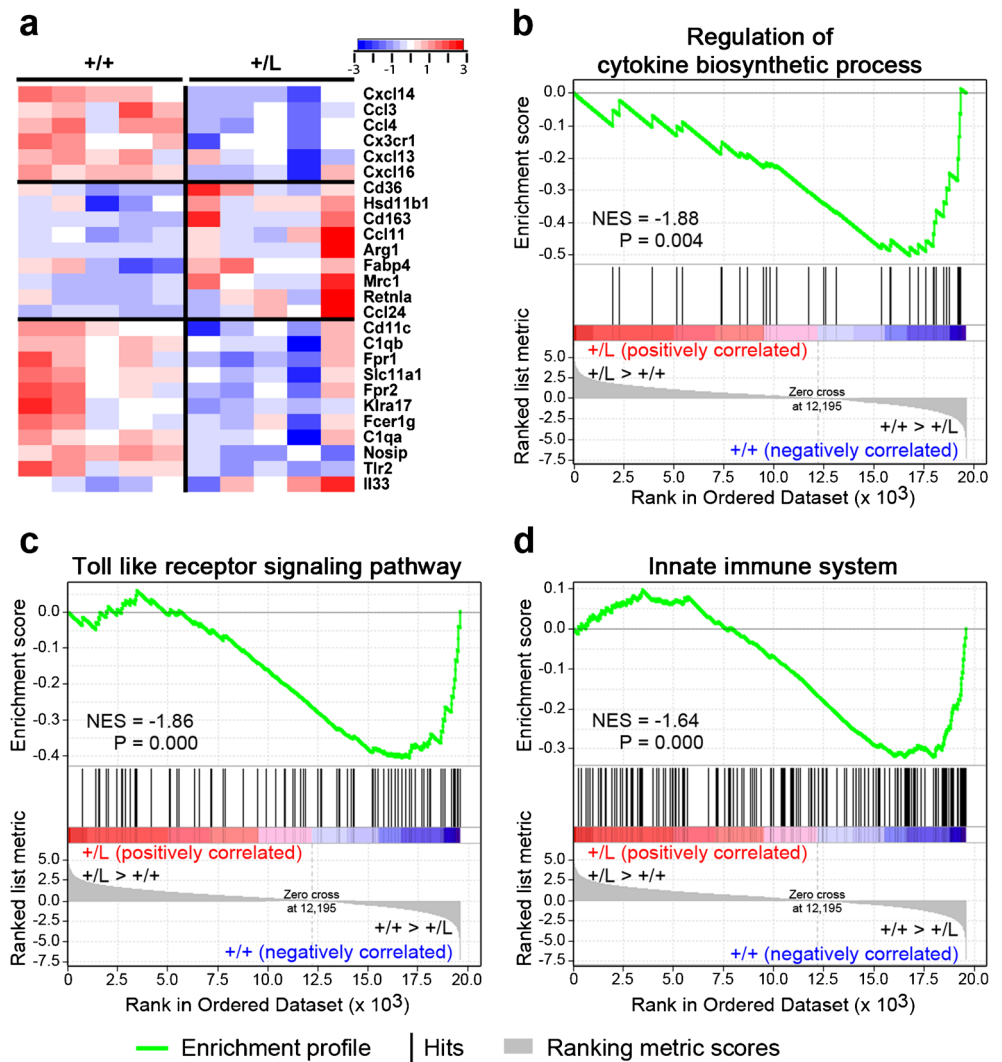
C/EBP β LIP is thought to antagonize the long isoforms C/EBP β LAP*/LAP, other C/EBP members, and some bZIP factors [36, 37]. Accordingly, four scenarios can be envisioned to describe the effect of C/EBP β LIP on gene expression: (i) C/EBP β LIP acts alone, (ii) C/EBP β LIP antagonizes other C/EBP family members or bZIP factors, (iii) C/EBP β LIP antagonizes the LAP*/LAP isoforms of C/EBP β , or (iv) ii and iii at the same time. C/EBP β LIP heterozygous mice can reflect all four modes of action while L/L mice can affect (i) and (ii) but not (iii) and (iv). C/EBP β

LIP heterozygous mice develop a broader tumor spectrum and higher percentage of tumor incidence than L/L mice, suggesting that the oncogenic action of C/EBP β LIP is mediated through antagonism of the C/EBP β LAP*/LAP isoforms. However, L/L mice die significantly earlier than +/L mice and tumor types that might develop later in life (e.g., requiring more oncogenic events) will not be found in L/L mice. Similarly, analysis of spontaneous tumor formation in p53-deficient mice showed a wider tumor spectrum in p53^{+/-} mice as compared to p53^{-/-} mice [28]. These data suggest that dosage effects of oncogenes or tumor suppressor genes may affect tumor development. Carcinogen-induced tumorigenesis in LIP ki mice or combination with other murine oncogenic models will help to resolve the underlying molecular events in future experiments. Surprisingly and in contrast to the *Cebpb*^{-/-} mice, L/L and +/L mice both do not show skin phenotypes, loss of hair, or reduced fat content (data not shown), suggesting that C/EBP β LIP isoform functions are not reflecting simple loss of function phenotypes and go beyond inhibition of C/EBP β LAP*/LAP or other C/EBPs members in the skin or fat. These observations support the notion that regulatory capacity by C/EBP β LIP is context dependent and more complex than the four possibilities of action, as noted above. Nevertheless, it was important to first analyze spontaneous tumor formation in LIP ki mice and here, our data revealed the oncogenic potential of C/EBP β LIP from its own locus in diverse tissues.

The long latency of tumor development in LIP ki mice suggests that additional oncogenic events are required in conjunction with C/EBP β LIP deregulation. Cooperation of several proto-oncogenes and loss of tumor suppressor functions is a common explanation of tumor development. However, tumorigenesis was not accelerated in compound mice heterozygous for p53 deletion and C/EBP β LIP deregulation, as compared to p53 heterozygous mice (data not shown). It therefore remains to be resolved which additional oncogenic events may accelerate tumor development in +/L or L/L mice.

The GSEA analyses indicated several altered pathways in lymphoma of LIP ki mice that relate to autonomous and non-cell autonomous effects on B cell lymphomagenesis. The structural features of *Cebpb* gene (lack of introns), however, render a conditional genetic approach rather difficult to experimentally resolve how C/EBP β LIP supports oncogenesis. In contrast to *Cebpa*, no mutational alterations within the *Cebpb* coding region that affect the isoform expression have yet been reported. However, deregulation of C/EBP β LIP expression may occur on the signaling/proteomic, rather than on the genomic level. In any case, our results show that tight regulation of the balance between long and truncated C/EBP β isoforms is important for preventing tumor formation. Accordingly, the data imply that translational deregulation may dysbalance C/EBP β isoform expression to contribute to tumorigenesis.

Fig. 5 Deregulated C/EBPβ LIP expression altered cytokines and chemokines expression levels in B-NHL of LIP ki mice. **a** Heat map showing deregulated expression of cytokines and chemokines in B-NHL of +/L mice in comparison to B-NHL of WT mice. +/+, *n*=5; +/L, *n*=5. **b–d** GSEA based on the comparison of B-NHL of +/L and WT mice for enrichment or depletion of regulation of cytokine biosynthetic process (**b**), Toll-like receptor signaling pathway (**c**), and innate immune system (**d**) associated genes. The normalized enrichment scores (NES) and *P* values are indicated in each plot. Note the negative NES values observed in all cases indicating a depletion of these gene sets in B-NHL of +/L mice compared to B-NHL of WT mice



Previously, we have shown that the mTOR-TORC1 inhibitor rapamycin restricts upregulation of C/EBPβ LIP expression and lymphoma xenograft growth [3, 14]. Moreover, a translational control defective C/EBPβ mutant phenocopied mTOR inhibition and mTOR target genes were found to be coregulated by C/EBPβ and thus identified C/EBPβ as an important mediator of mTOR functions [9, 10]. Activation of mTOR promotes protein biosynthesis, translation reinitiation, M2 polarization, cell survival, and tumorigenesis [38, 39]. Deregulated mTOR signaling is evident in lymphomagenesis, and leukemogenesis and development of therapeutic strategies based on mTOR inhibition are currently under investigation [40, 40]. Lymphoma cells of C/EBPβ LIP heterozygous mice showed enrichment of rapamycin-sensitive genes, including FABP4 and adipokines that are thought to play an important role in tumorigenesis [41]. In addition, eIF-4E, a key factor of translation initiation that is regulated by the

mTOR-sensitive 4EBPs also upregulates C/EBPβ LIP expression and promotes neoplasia [3, 42]. Our data therefore imply that pharmacologic interference with uORF-mediated C/EBPβ LIP translation initiation control may help to reestablish the balance between C/EBPβ isoforms and oppose deregulated mTOR signaling.

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