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C/EBPβΔuORF mice—a genetic model for uORF-mediated translational control in mammals

Klaus Wethmar, Valérie Bégay, Jeske J. Smink, Katrin Zaragoza, Volker Wiesenthal, Bernd Dörken, Cornelis F. Calkhoven, and Achim Leutz

1Max Delbrück Center for Molecular Medicine, D-13092 Berlin, Germany; 2Charité, Campus Virchow Klinikum, University Medicine Berlin, D-13353 Berlin, Germany; 3Leibniz Institute for Age Research–Fritz Lipmann Institute, D-07745 Jena, Germany; 4Department of Biology, Humboldt-University, D-10115 Berlin, Germany

Upstream ORFs (uORFs) are translational control elements found predominantly in transcripts of key regulatory genes. No mammalian genetic model exists to experimentally validate the physiological relevance of uORF-regulated translation initiation. We report that mice deficient for the CCAAT/enhancer-binding protein β (C/EBPβ) uORF initiation codon fail to initiate translation of the autoantagonistic LIP (liver inhibitory protein) C/EBPβ isoform. C/EBPβΔuORF mice show hyperactivation of acute-phase response genes, persistent repression of E2F-regulated genes, delayed and blunted S-phase entry of hepatocytes after partial hepatectomy, and impaired osteoclast differentiation. These data and the widespread prevalence of uORFs in mammalian transcriptomes suggest a comprehensive role of uORF-regulated translation in physiological processes, including metabolism, innate immunity, liver development, and regeneration (Tagana et al. 1995; Greenbaum et al. 1998; Ramji and Foka 2002).

The transcription factor CCAAT/enhancer-binding protein β [C/EBPβ] exerts important functions in many physiological processes, including metabolism, innate immunity, liver development, and regeneration (Tagana et al. 1995; Greenbaum et al. 1998; Ramji and Foka 2002). The C/EBPβ gene lacks introns, yet three N-terminal different isoforms (termed LAP* [liver-activating protein*], LAP, and LIP [liver inhibitory protein]) are translated from three consecutive in-frame AUG codons in a single transcript (Fig. 1A; Descombes and Schibler 1991). The truncated isoform LIP is devoid of N-terminal trans-activating domains but retains DNA-binding capacity and acts as a competitive inhibitor of the LAP and LAP* isoforms. Previous mutational analysis and tissue culture experiments suggested that translation of the conserved out-of-frame C/EBPβ uORF restrains initiation of LAP and causes repression of ribosomal scanning and reinitiation at the downstream LIP start site (Fig. 1B; Raught et al. 1996; Lincoln et al. 1998; Calkhoven et al. 2000).

Results and Discussion

To determine the physiological importance of uORF-mediated translational control, recombinant mice were generated by introduction of an ATG-to-TTG point mutation at the C/EBPβ uORF translational initiation site [Fig. 1B; Supplemental Fig. S2]. The C/EBPβΔuORF mutation was designed to abrogate uORF initiation without altering the amino acid sequence of C/EBPβ. A C/EBPβ wild-type knock-in control strain [C/EBPβWT] was generated and analyzed in parallel to exclude potential artifacts caused by the gene targeting approach. Throughout the experiments, no differences were detected between C/EBPβWT and parental wild-type mice. Offspring of heterozygous C/EBPβΔuORF matings showed the expected Mendelian ratio (Supplemental Table S1). Heterozygous C/EBPβΔuORF females were fertile, gave birth to normal size litters (eight out of eight females tested), and showed intact mammary gland development and function (Robinson et al. 1998; Scagroves et al. 1998; Supplemental Figs. S3A, S4).

Bacterial lipopolysaccharide [LPS] is a major inducer of acute-phase response mediated by C/EBPβ in the liver (Poli 1998), and has been shown to enhance expression of the truncated C/EBPβ isoform LIP (Timchenko et al. 2005). In livers of C/EBPβWT mice, LIP was strongly induced after LPS administration, whereas C/EBPβΔuORF mice failed to express high levels of LIP [Fig. 1C]. Likewise, lung, spleen, and white adipose tissue of C/EBPβWT mice displayed reduced expression of LIP after LPS treatment as compared with C/EBPβWT tissues (Supplemental Fig. S5). Failure to induce LIP was also observed in C/EBPβΔuORF mouse embryonic fibroblasts (MEFs) [Fig. 1D] and was associated with superactivation of a C/EBPβ-responsive luciferase reporter [Fig. 1E], suggesting a lack of trans-repressive function of LIP. Hence, genetic ablation...
isoforms showed strongly enhanced osteoclast differentiation. Ectopic expression of LIP inhibited osteoclast differentiation (Smink et al. 2009), suggesting that an increase of the LAP/LIP isoform ratio in C/EBPb uORF mice would also inhibit osteoclastogenesis. In tibiae of C/EBPbΔuORF mice, we observed a reduction in osteoclast size and number (Fig. 2A), which was accompanied by an increase in thickness of bone trabeculae and bone volume (Supplemental Fig. S6). Bone marrow cell cultures from C/EBPbΔuORF mice formed fewer and smaller osteoclasts as compared with C/EBPbWT mice and failed to express LIP (Fig. 2B, C). C/EBPbΔuORF osteoclasts showed increased expression of the transcription factor MafB (Fig. 2D), a previously identified target of LAP and a repressor of osteoclastogenesis (Smink et al. 2009). MafB inhibits a number of osteoclastic genes, including Nfatc1, Oscar, Atp6v0d2, DC-STAMP, and TRACP (Kim et al. 2007, 2008; Smink et al. 2009). Transcript levels of these osteoclast markers were found to be reduced in C/EBPbΔuORF osteoclasts (Fig. 2E), which express isoforms of the MaßB-independent c-Fos gene (Kim et al. 2007) was not affected. These data suggest that the abrogation of C/EBPb uORF-mediated translational control, and the

**Figure 1.** Genetic ablation of cis-regulatory translational control by the C/EBPb uORF. (A) Three protein isoforms [LAP* (38 kDa), LAP (35 kDa), and LIP (20 kDa)] are translated from consecutive in-frame initiation codons in the same transcript (Descombes and Schibler 1991). The C/EBPb mRNA contains a conserved cis-regulatory small uORF (30 base pairs [bp], orange) terminating 4 bp upstream of the LAP initiation site in a different reading frame. (bd) Binding; (pA) polyA tail. (b) Translation of the uORF serves to strip ribosomes from their initiating Met-tRNAMet (green to white) and prevents initiation at the proximate LAP initiation codon. Upon reloading of ribosomes with the ternary eIF2–GTP–Met-tRNAMet complex (white to green), translation reinitiation from the downstream AUG codon generates LIP. In C/EBPbΔuORF mice, an A-to-U point mutation was designed to abrogate ribosomal initiation at the uORF start codon without changing the amino acid sequence of the C/EBPb isoforms. Most ribosomes will thus initiate at the LAP AUG instead. (Display of LAP* translation was omitted for simplicity. For details on alternative start site selection, see Supplemental Fig. S1.) (C) Upon i.p. injection of LPS, LIP is strongly induced in C/EBPbWT (WT) but not in C/EBPbΔuORF livers (Δ). (d) Hours of LPS treatment; (α-tub.) α-tubulin; (k.o.) lysate of C/EBPb knockout mouse. (D) In MEFs, LPS induces LIP expression in C/EBPbWT but not in C/EBPbΔuORF cells. (E) Representative luciferase reporter assay (n = 3) demonstrating increased luciferase reporter activity [Luc.] in C/EBPbΔuORF (open triangles) as compared with C/EBPbWT (black squares) MEFs at indicated times after LPS treatment. Error bars show SEM.  

of the C/EBPb uORF in mice abolishes the inducible expression of LIP and validates the functional importance of the C/EBPb uORF as a translational cis-regulatory element in the animal.

Recently, we showed that the long and truncated C/EBPb isoforms opposingly regulate the differentiation of bone-resorbing osteoclasts (Smink et al. 2009). C/EBPbLIP mice that express LIP only and not the long C/EBPb
resulting increase in the LAP/LIP ratio, constrains osteoclast differentiation by enhancing the expression of cFos.

Since C/EBPβ is an important regulator of liver regeneration, acute-phase response, and Interleukin-6 (IL-6) expression (Screpanti et al. 1995; Greenbaum et al. 1998; Poli 1998), we implemented partial hepatectomy (PH) to analyze the consequences of the C/EBPβΔuORF mutation in this physiological context. C/EBPβΔuORF mice failed to induce expression of the truncated LIP isofrom throughout the 72-h observation period after PH (Fig. 3A), while LIP was strongly induced in a two-wave kinetic in regenerating livers of C/EBPβWT animals, suggesting consecutive functions of LIP in the course of liver regeneration. After PH, IL-6 serum levels of C/EBPβΔuORF mice rose higher as compared with control animals (Fig. 3B), reaching a 4.7-fold difference after 3 h (1254 ± 265 vs. 263 ± 49 pg/mL, n = 6, P < 0.01) and a 3.3-fold difference at the peak of wild-type expression 6 h after surgery (1578 ± 132 vs. 472 ± 93 pg/mL, n = 6, P < 0.01). IL-6 signaling is known to rapidly confer activating phosphomodifications to both C/EBPβ and STAT3 transcription factors (Akira 1997), resulting in synergistic induction of type I acute-phase response genes (Alonzi et al. 2001). Real-time PCR analysis of known acute-phase response C/EBPβ target genes revealed consistently increased transcription of serum amyloid A1 (Saa1), α-1 antitrypsin (Aat), haptoglobin (Hp), and hemopexin (Hp), ranging from 1.2-fold to 8.0-fold in hepatectomized C/EBPβΔuORF as compared with C/EBPβWT mice (Fig. 3C). Maxima of enhanced expression of Saa1, Aat, and Hp in C/EBPβΔuORF mice correlated with the peak of LAP expression at 6 h after surgery (Fig. 3A). Together with the superactivation of the C/EBP-responsive reporter construct in C/EBPβΔuORF MEFs, these data suggest that uORF-mediated induction of LIP serves to restrict the trans-activation of early acute-phase response genes.

In an in vitro proliferation assay, reduced expansion of C/EBPβΔuORF cultures [n = 5 independent embryos per genotype, [*] P < 0.01]. (B) Quantification of BrdU-labeled hepatocyte nuclei (2-h pulse-labeling) in liver sections showing a reduced proportion of hepatocytes in S phase of C/EBPβΔuORF (open triangles) as compared with C/EBPβWT (black bars) and C/EBPβΔuORF (gray bars) livers at 6 and 48 h after PH [n = 8, [*] P < 0.01; n = 7, [*] P < 0.05 vs. wild type, respectively]. (C) BrdU immunofluorescence stainings of C/EBPβWT, C/EBPβΔuORF, and C/EBPβΔuORF liver sections 36 h after PH. Bars, 100 μm. (D) Real-time PCR analysis showing reduced mRNA contents of CcnA1, CcnA2, CcnB1, CcnE1, CcnE2, and Pena in C/EBPβΔuORF (open bars) as compared with C/EBPβWT (black bars) and C/EBPβΔuORF (gray bars) livers at indicated times after PH. (n = 6, [*] P < 0.05, [**] P < 0.01 vs. wild type). (n.d.) Not determined. Error bars show SEM.

Figure 3. The C/EBPβΔuORF mutation causes superinduction of C/EBPβ target genes. (A) Induction of LIP in C/EBPβWT livers upon PH is abolished in C/EBPβΔuORF animals. (B) ELISA showing elevated average levels of serum IL-6 at 3 h and 6 h after PH in C/EBPβΔuORF (open triangles) as compared with C/EBPβWT (black squares) animals [n = 6, [*] P < 0.01]. (C) Real-time PCR analysis demonstrating elevated mRNA contents of acute-phase response genes in C/EBPβΔuORF (open bars) as compared with C/EBPβWT [black bars] livers at indicated times after PH [n = 6, [*] P < 0.05; [**] P < 0.01]. Error bars show SEM.

Figure 4. Cell proliferation defect in C/EBPβΔuORF mice. (A) In vitro proliferation assay demonstrating reduced expansion of C/EBPβΔuORF (open triangles) as compared with C/EBPβWT (black squares) MEF cultures [n = 5 independent embryos per genotype, [*] P < 0.01]. (B) Quantification of BrdU-labeled hepatocyte nuclei (2-h pulse-labeling) in liver sections showing a reduced proportion of hepatocytes in S phase of C/EBPβΔuORF (open triangles) as compared with C/EBPβWT (black bars) and C/EBPβΔuORF (gray bars) livers at 6 and 48 h after PH [n = 8, [*] P < 0.01; n = 7, [*] P < 0.05 vs. wild type, respectively]. (C) BrdU immunofluorescence stainings of C/EBPβWT, C/EBPβΔuORF, and C/EBPβΔuORF liver sections 36 h after PH. Bars, 100 μm. (D) Real-time PCR analysis showing reduced mRNA contents of CcnA1, CcnA2, CcnB1, CcnE1, CcnE2, and Pena in C/EBPβΔuORF (open bars) as compared with C/EBPβWT (black bars) and C/EBPβΔuORF (gray bars) livers at indicated times after PH. (n = 6, [*] P < 0.05, [**] P < 0.01 vs. wild type). (n.d.) Not determined. Error bars show SEM.
day 5 of the experiment (9.2 ± 0.2 vs. 11.9 ± 0.5 × 10^6 per well, n = 5, P < 0.01) as compared with C/EBPβ WT MEFs. To examine whether the C/EBPβ WT mutation also affected cell proliferation in mice, we compared liver regeneration properties of C/EBPβuORF, C/EBPβ WT, and C/EBPβα decisions (C/EBPβ expression control) in Supplemental Fig. S7). Hepatocytes in regenerating livers of C/EBPβ WT mice entered the cell cycle later and at lower frequency as compared with C/EBPβ WT animals [Fig. 4B,C]. S-phase labeling of liver cells by 5-Bromo-2-deoxyuridine (BrdU) revealed a 9.3-fold reduction in the frequency of BrdU-positive C/EBPβuORF hepatocytes at 36 h [1.2 ± 0.5% vs. 11.6 ± 2.8%, n = 8, P < 0.01] and a 1.9-fold reduction at 48 h after surgery (28.9% ± 4.0% vs. 54.9% ± 3.8%, n = 7, P < 0.01). At the same times, regenerating C/EBPβLIP livers contained similar numbers of BrdU-positive hepatocytes as compared with C/EBPβ WT (36 h: 11.5% ± 0.4%, n = 5; 48 h: 56.9% ± 2.1%, n = 4). Virtually no BrdU incorporation was observed in hepatocytes of sham-operated animals at 48 h after PH [n = 3] (data not shown). Transcript levels of cyclin A1 [CcnA1], CcnA2, CcnB1, CcnE1, CcnE2, and proliferating cell nuclear antigen [PcnA] were induced at 36 h after PH in C/EBPβ WT and C/EBPβLIP livers, but remained significantly lower in C/EBPβuORF animals [Fig. 4D]. Twelve hours later, the expression of the cyclins and PcnA were similar in the three genotypes [data not shown], suggesting that re-entry of C/EBPβuORF hepatocytes into the cell cycle was impeded but not abolished by the compromised induction of LIP. Similar recovery of liver weight in C/EBPβ WT and C/EBPβuORF mice, accompanied by an increased hepatocyte volume in C/EBPβuORF livers [Supplemental Fig. S8], suggested that enhanced hepatocyte hypertrophy compensated for the blunted S-phase entry to restore adequate liver/body weight ratios.

To further characterize the altered dynamics of cell cycle entry in regenerating C/EBPβuORF livers, we performed a genome-wide microarray expression analysis at 36 h after PH. A total number of 546 underrepresented transcripts [392 annotated genes] and 266 overrepresented transcripts [161 annotated genes] were identified in regenerating C/EBPβuORF as compared with C/EBPβ WT livers [Fig. 5A; Supplemental Table S2]. Comparison of all deregulated transcripts to a database of cell cycle-associated genes [http://www.geneontology.org] resulted in 191 matches, of which 99% (189 matches) grouped to the underrepresented fraction [Fig. 5A; Supplemental Table S3]. The microarray analysis results were validated for a selection of transcripts on the mRNA (Fig. 4D) and/or the protein level [Supplemental Fig. S9]. The high proportion of underrepresented cell cycle genes at 36 h after PH verified the immunohistochemically detected reduction in hepatocyte S-phase entry in C/EBPβuORF mice on a transcriptional level, and implied a regulatory function of the C/EBPβ LAP/LIP isoform ratio.

C/EBP transcription factors are known to affect the expression of cell cycle regulatory genes controlled by E2F transcription factors [Sebastian and Johnson 2006; Nerlov 2007]. Full-length C/EBPα [p42], but not the N-terminally truncated isoform [p30], acts as a cell cycle inhibitor by repressing E2F target genes [Slomiany et al. 2000, Porse et al. 2001; Iakova et al. 2003]. For C/EBPβ, isoform-specific data on E2F corregulation is scarce and suggested a corepressive function of LAP [Sebastian et al. 2005]. A comparison of deregulated cell cycle genes in regenerating C/EBPβuORF liver to previously identified E2F targets [Ishida et al. 2001; Ren et al. 2002; Bracken et al. 2004] revealed that at least 42% of them were known E2F target genes. Chromatin immunoprecipitation (ChIP) analysis performed 36 h after PH showed that both E2F3 and C/EBPβ were associated with promoters of underrepresented E2F target genes in regenerating liver [E2F1, Rb1 [retinoblastoma-like 1], CcnA2, CcnE1, Cdc2 [cell division cycle-associated 2], Cdc25, Mcm3 [mini-chromosome maintenance-deficient 3], Mcm6, and Plk1 [Polo-like kinase 1]] [Fig. 5B]. At the same time, C/EBPα showed little or no association with these E2F target gene promoters. Furthermore, transient down-regulation of transcript and protein levels of C/EBPα after PH [Supplemental Fig. S10] suggested a predominant role for C/EBPβ in the coregulation of many E2F target genes in cycling hepatocytes. To examine the effect of individual C/EBPβ isoforms on E2F coregulation, we used an E2F-responsive luciferase reporter construct that has been employed previously to address the mechanism of C/EBP-mediated E2F repression [Porse et al. 2001]. Luciferase activity induced by ectopic expression of

**Figure 5.** The C/EBPβuORF mutation causes repression of E2F target genes. [A] Graphic representation of a genome-wide microarray expression analysis comparing transcript levels in C/EBPβ WT and C/EBPβuORF liver at 36 h after PH. [B] Representative ChIP assay on C/EBPβ WT liver chromatin showing the association of E2F3, C/EBPα, and C/EBPβ to indicated gene promoters in regenerating liver at 36 h after PH [n = 2]. (C) Luciferase reporter assay demonstrating the repressive function of long [black bars], but not of truncated [open bars] C/EBPα and C/EBPβ isoforms on the pGL3TATAbasic-6xE2F reporter construct [n = 3]. [Luc] Luciferase activity; [p42 and p30] long and truncated C/EBPα isoforms. [D] Luciferase reporter assay with constant, immediately repressive C/EBPβ p42 expression [luciferase activity set to 0.5] showing the corepressive function of LAP and LIP [black bars] and the derepressing function of LIP [open bars] on the same E2F-responsive reporter construct as used in C [n = 3]. Error bars show SEM.
the transcription factors E2F1 and DP1 (dimerization partner 1, conferring full activity to E2F1) was proportionally repressed by increasing amounts of coexpressed p42, LAP*, or LAP, but remained unaffected by coexpression of LAP or LIP [Fig. 5C]. Importantly, the inhibition of E2F activity by p42 was effectively relieved by coexpression of LIP, whereas increasing amounts of LAP* or LAP further repressed reporter activity [Fig. 5D].

Liver regeneration defects observed in C/EBPβ knockout mice have been attributed to a lack of coactivating C/EBPβ function on E2F target genes (Greenbaum et al. 1998; Wang et al. 2007). This interpretation contrasts data showing E2F-repressive functions of LAP in vitro (Sebastian et al. 2005) and LAP-mediated retardation of hepatocyte cell cycle entry after PH in mice (Luedde et al. 2004). Our observations of impaired cell cycle entry in C/EBPβuORF livers and the rescue of this phenotype in C/EBPβ−/− mice suggest that long C/EBPβ isoforms are dispensable for accurate hepatocyte S-phase entry. The data presented here imply a model in which uORF-mediated induction of LIP is required to overcome repression of E2F targets by long C/EBPβ and C/EBPβ isoforms to facilitate rapid cell cycle entry during liver regeneration.

The analysis of the C/EBPβuORF mice proves the physiological relevance of uORF-mediated translational control in mammals. We note that low amounts of LIP can be detected in C/EBPβuORF mice, which might originate from leaky ribosomal scanning over both the LAP* and LAP start codons [Supplemental Fig. S1] or from partial proteolytic cleavage [Baer and Johnson 2000]. Nevertheless, the lack of a functional C/EBPβ uORF start codon results in the inability to induce LIP expression under inflammatory conditions, as well as during differentiation and regeneration processes. aberrant protein expression caused by defective translational control is increasingly recognized as a pathophysiological mechanism in the etiology of human diseases [Scheper et al. 2007]. Specifically, mutations affecting uORF-mediated translational control have been connected to the development of diseases such as hereditary thrombocytopenia [Wiestner et al. 1998], familial cutaneous melanoma [Liu et al. 1999], or Marie Unna hereditary hypotrichosis [Wen et al. 2009]. The high prevalence of uORFs in human transcripts (35%–49%) implies a comprehensive, yet underestimated, cis-regulatory function in adjusting protein expression [Iacono et al. 2005; Calvo et al. 2009]. Future studies will have to address to what extent aberrant uORF-mediated translational control accounts for the development of disease, and how it can be targeted by therapeutic intervention.

Materials and methods

**Generation of C/EBPβuORF and C/EBPβWT mice**

Mutant (C/EBPβuORF) and control (C/EBPβWT) mice were generated by homologous recombination according to standard protocols [Supplemental Fig. S2]. The neomycin cassette was removed by crossing C/EBPβuORF and C/EBPβWT mice to the Cre-deleter strain [Schwenk et al. 1995], and the new strains were kept in a 129Ola × C57Bl/6 background. Female and male mice showed the same phenotype and were analyzed as one group. Mice were provided with standard mouse diet and water ad libitum on a 12-h light–dark cycle. All procedures and animal experiments were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee.

Additional methods can be found in the Supplemental Material.

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**References**


