C/EBPβ$^{\Delta uORF}$ mice—a genetic model for uORF-mediated translational control in mammals

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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/EBPβ$^{\Delta uORF}$ 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 (patho)physiology.

Supplemental material is available at http://www.genesdev.org.

Translational *cis*-regulatory upstream ORFs (uORFs) are found in the 5′ mRNA regions of numerous eukaryotic transcripts and are considered to regulate protein expression by controlling translation reinitiation at downstream initiation codons or by activating the nonsense-mediated mRNA decay pathway (Morris and Geballe 2000). The frequency of conserved uORFs (Iacono et al. 2005; Calvo et al. 2009) and their predominant prevalence in transcripts of key regulatory genes of growth, differentiation, and proliferation (Kozak 1987) suggest an impor-

tant function of uORF-mediated translational control in mammals. Nevertheless, an experimental genetic model to examine the physiological relevance of uORF-regulated translation has not been established.

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 (Tanaka 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 resumption 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/EBPβ$^{\Delta uORF}$ 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/EBPβ^{WT}) 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/EBPβ^{WT} and parental wild-type mice. Offspring of heterozygous C/EBPβ$^{\Delta uORF}$ matings showed the expected Mendelian ratio (Supplemental Table S1). Homozygous C/EBPβ$^{\Delta uORF}$ mice showed normal weight gain and no overt developmental defects or premature death (Supplemental Fig. S3). In contrast to C/EBPβ knockout animals, homozygous C/EBPβ$^{\Delta uORF}$ 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; Seagroves 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/EBPβ^{WT} mice, LIP was strongly induced after LPS administration, whereas C/EBPβ$^{\Delta uORF}$ mice failed to express high levels of LIP (Fig. 1C). Likewise, lung, spleen, and white adipose tissue of C/EBPβ$^{\Delta uORF}$ mice displayed reduced expression of LIP after LPS treatment as compared with C/EBPβ^{WT} tissues (Supplemental Fig. S5). Failure to induce LIP was also observed in C/EBPβ$^{\Delta uORF}$ mouse embryo fibroblasts (MEFs) (Fig. 1D) and was associated with superactivation of a C/EBP-responsive luciferase reporter (Fig. 1E), suggesting a lack of *trans*-repressive function of LIP. Hence, genetic ablation
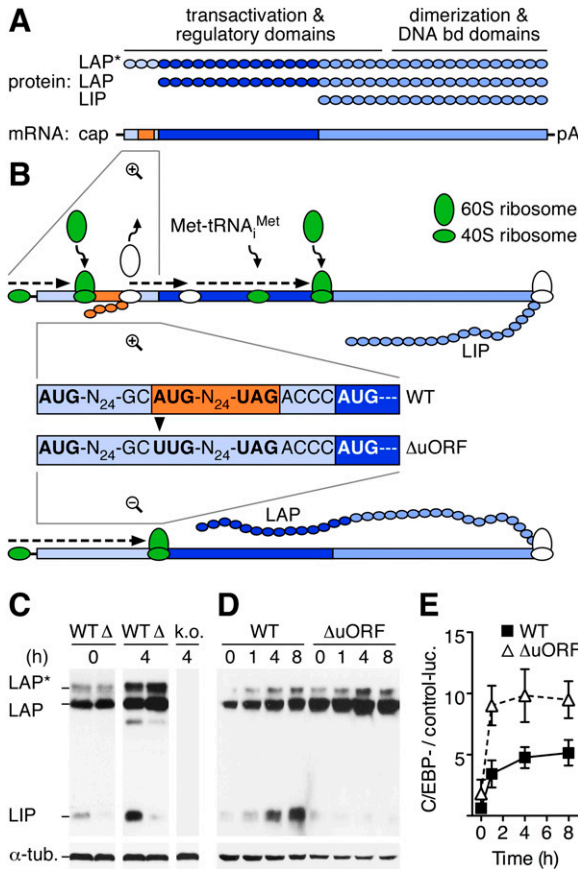
Figure 1. Genetic ablation of *cis*-regulatory translational control by the C/EBPβ uORF. (*A*) Three protein isoforms (LAP* [38 kDa], LAP [35 kDa], and LIP [20 kDa]) are translated from consecutive in-frame initiation codons in the same transcript (Descombes and Schibler 1991). The C/EBPβ mRNA contains 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) poly(A) tail. (*B*) Translation of the uORF serves to strip ribosomes from their initiating Met-tRNA$_i^{Met}$ (green to white) and prevents initiation at the proximate LAP initiation codon. Upon reloading of ribosomes with the ternary eIF2–GTP–Met-tRNA$_i^{Met}$ complex (white to green), translation reinitiation from the downstream AUG codon generates LIP. In C/EBPβ$^{\Delta uORF}$ 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/EBPβ 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/EBPβ^{WT} (WT) but not in C/EBPβ$^{\Delta uORF}$ livers (Δ). (h) Hours of LPS treatment; (α-tub.) α-tubulin; (k.o.) lysate of C/EBPβ knockout mouse. (*D*) In MEFs, LPS induces LIP expression in C/EBPβ^{WT} but not in C/EBPβ$^{\Delta uORF}$ cells. (*E*) Representative luciferase reporter assay (*n* = 3) demonstrating increased luciferase reporter activity (luc.) in C/EBPβ$^{\Delta uORF}$ (open triangles) as compared with C/EBPβ^{WT} (black squares) MEFs at indicated times after LPS treatment. Error bars show SEM.

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

Recently, we showed that the long and truncated C/EBPβ isoforms opposingly regulate the differentiation of bone-resorbing osteoclasts (Smink et al. 2009). C/EBPβ^{LIP} mice that express LIP only and not the long C/EBPβ

isoforms showed strongly enhanced osteoclast differentiation. Ectopic expression of LAP inhibited osteoclast differentiation (Smink et al. 2009), suggesting that an increase of the LAP/LIP isoform ratio in C/EBPβ$^{\Delta uORF}$ mice would also inhibit osteoclastogenesis. In tibiae of C/EBPβ$^{\Delta uORF}$ 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/EBPβ$^{\Delta uORF}$ mice formed fewer and smaller osteoclasts as compared with C/EBPβ^{WT} and failed to express LIP (Fig. 2B,C). C/EBPβ$^{\Delta uORF}$ 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/EBPβ$^{\Delta uORF}$ osteoclasts (Fig. 2E), while expression of the MafB-independent *c-Fos* gene (Kim et al. 2007) was not affected. These data suggest that the abrogation of C/EBPβ uORF-mediated translational control, and the
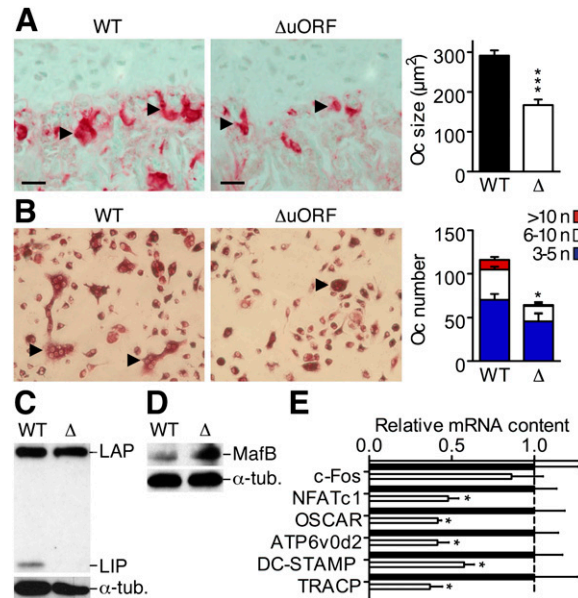

Figure 2. The C/EBPβ$^{\Delta uORF}$ mutation impairs osteoclast differentiation. (*A*) Tibia sections showing tartrate-resistant acid phosphatase (TRACP)-stained osteoclasts (red staining, light-green counterstain) in C/EBPβ$^{\Delta uORF}$ as compared with C/EBPβ^{WT} mice. (Arrowheads) Multinucleated osteoclasts; bars, 50 μm. The bar graph displays average osteoclast sizes as determined from six mice per genotype at 8 wk of age. (*B*) TRACP staining (red) showing osteoclast differentiation of bone marrow-derived precursors of C/EBPβ$^{\Delta uORF}$ and C/EBPβ^{WT} mice after 6 d in culture with M-CSF and RANK-L (*n* = 6). (Arrowheads) Multinucleated osteoclasts. The bar graph displays the differential quantification of osteoclasts by the number of nuclei (*n*) per cell. (*C*) Immunoblot analysis showing LIP expression in C/EBPβ^{WT} but not in C/EBPβ$^{\Delta uORF}$ osteoclasts at day 2 of culture. (*D*) Immunoblot analysis showing increased MafB protein in C/EBPβ$^{\Delta uORF}$ as compared with C/EBPβ^{WT} osteoclasts. (*E*) Real-time PCR analysis showing decreased expression of MafB-regulated osteoclast markers in C/EBPβ$^{\Delta uORF}$ (open bars) as compared with C/EBPβ^{WT} (black bars) osteoclasts. Normalized to Gapdh (glyceraldehyde-3-phosphate-dehydrogenase) and presented relative to C/EBPβ^{WT} (set to 1, dashed line). Error bars show SEM; (*) $P < 0.05$; (***) $P < 0.001$.

resulting increase in the LAP/LIP ratio, constrains osteoclast differentiation by enhancing the expression of MafB.

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/EBPβ^{ΔuORF} mutation in this physiological context. C/EBPβ^{ΔuORF} mice failed to induce expression of the truncated LIP isoform 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/EBPβ^{WT} animals, suggesting consecutive functions of LIP in the course of liver regeneration. After PH, IL-6 serum levels of C/EBPβ^{ΔuORF} 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* (*Hpx*), ranging from 1.2-fold to 8.0-fold in hepatectomized C/EBPβ^{ΔuORF} as compared with C/EBPβ^{WT} mice (Fig. 3C). Maxima of en-
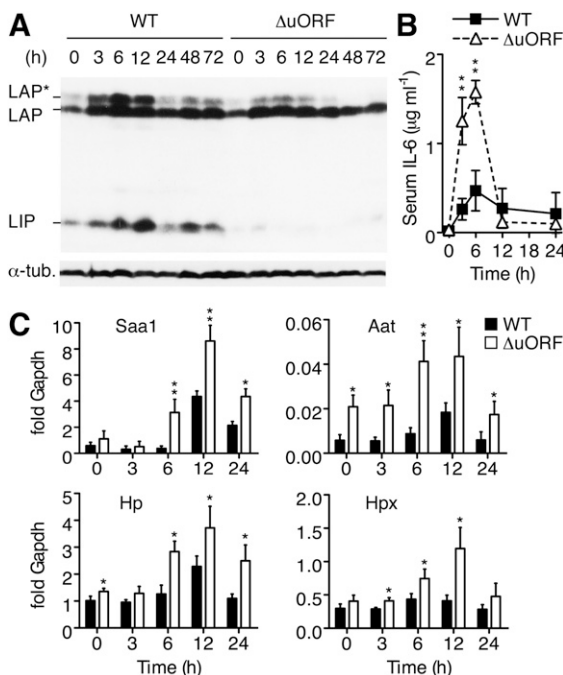

Figure 3. The C/EBPβ^{ΔuORF} mutation causes superinduction of C/EBPβ target genes. (*A*) Induction of LIP in C/EBPβ^{WT} livers upon PH is abolished in C/EBPβ^{ΔuORF} animals. (*B*) ELISA showing elevated average levels of serum IL-6 at 3 h and 6 h after PH in C/EBPβ^{ΔuORF} (open triangles) as compared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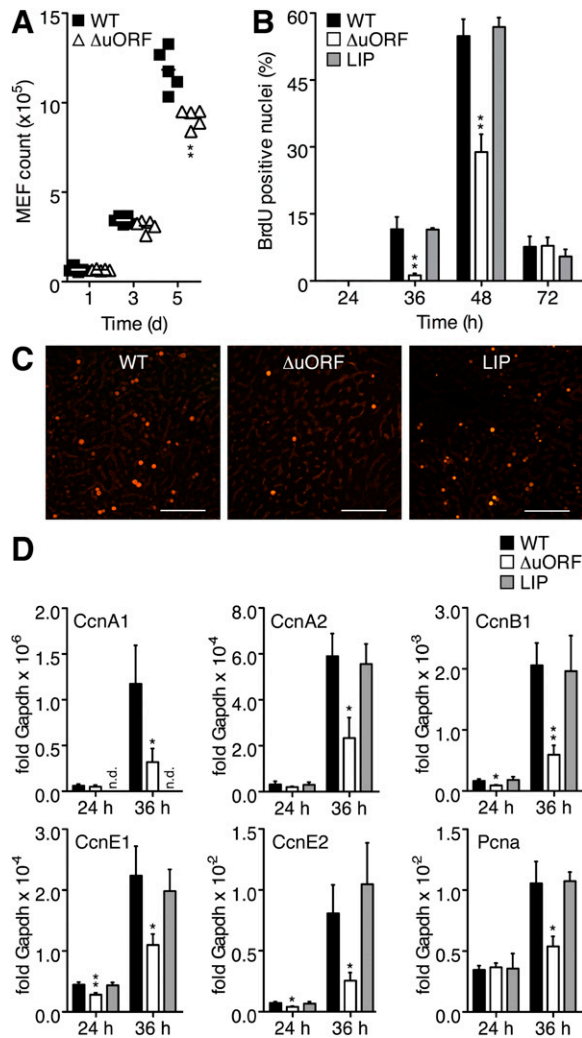

Figure 4. Cell proliferation defect in C/EBPβ^{ΔuORF} mice. (*A*) In vitro proliferation assay demonstrating reduced expansion of C/EBPβ^{ΔuORF} (open triangles) as compared with C/EBPβ^{WT} (black squares) MEF cultures ($n = 5$ independent embryos per genotype; [**] $P < 0.01$). (*B*) Quantification of BrdU-labeled hepatocyte nuclei (2-h pulse-labeling) in liver sections showing a reduced proportion of hepatocytes in S phase in C/EBPβ^{ΔuORF} (open bars) as compared with C/EBPβ^{WT} (black bars) and C/EBPβ^{LIP} (gray bars) livers at 36 and 48 h after PH ($n = 8$, [***] $P < 0.001$; $n = 7$, [*] $P < 0.05$ vs. wild type, respectively). (*C*) BrdU immunofluorescence stainings of C/EBPβ^{WT}, C/EBPβ^{ΔuORF}, and C/EBPβ^{LIP} 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 Pcna in C/EBPβ^{ΔuORF} (open bars) as compared with C/EBPβ^{WT} (black bars) and C/EBPβ^{LIP} (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.

hanced expression of *Saa1*, *Aat*, and *Hp* in C/EBPβ^{ΔuORF} 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/EBPβ^{ΔuORF} 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/EBPβ^{ΔuORF} MEF cultures became evident at day 3 (Fig. 4A) and resulted in significantly lower cell numbers at

day 5 of the experiment $(9.2 \pm 0.2$ vs. $11.9 \pm 0.5 \times 10^5$ per well, $n = 5$, $P < 0.01$) as compared with C/EBPβ^{WT} MEFs. To examine whether the C/EBPβ^{ΔuORF} mutation also affected cell proliferation in mice, we compared liver regeneration properties of C/EBPβ^{ΔuORF}, C/EBPβ^{WT}, and C/EBPβ^{LIP} animals (C/EBPβ expression control in Supplemental Fig. S7). Hepatocytes in regenerating livers of C/EBPβ^{ΔuORF} 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 proportion of BrdU-positive C/EBPβ^{ΔuORF} hepatocytes at 36 h $(1.2\% \pm 0.5\%$ vs. $11.6\% \pm 2.8\%$, $n = 8$, $P < 0.01)$ and a 1.9-fold reduction at 48 h after surgery $(28.9\% \pm 4.0\%$ vs. $54.9\% \pm 3.8\%$, $n = 7$, $P < 0.01)$. At the same times, regenerating C/EBPβ^{LIP} livers contained similar numbers of BrdU-positive hepatocytes as compared with C/EBPβ^{WT} (36 h: $11.5\% \pm 0.4\%$, $n = 5$; 48 h: $56.9\% \pm 2.1\%$, $n = 4$). 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/EBPβ^{LIP} livers, but remained significantly lower in C/EBPβ^{ΔuORF} 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/EBPβ^{ΔuORF} hepatocytes into the cell cycle was impaired but not abolished by the compromised induction of LIP. Similar recovery of liver weight in C/EBPβ^{WT} and C/EBPβ^{ΔuORF} mice, accompanied by an increased hepatocyte volume in C/EBPβ^{ΔuORF} 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/EBPβ^{ΔuORF} 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/EBPβ^{ΔuORF} 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/EBPβ^{ΔuORF} 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 coregulation is scarce and suggested a corepressive function of LAP (Sebastian et al. 2005). A comparison of deregulated cell cycle genes in regenerating C/EBPβ^{ΔuORF} 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, Rbl1 [retinoblastoma-like 1], CcnA2, CcnE1, Cdc2 [cell division cycle-associated 2], Cdc25, Mcm3 [minichromosome 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
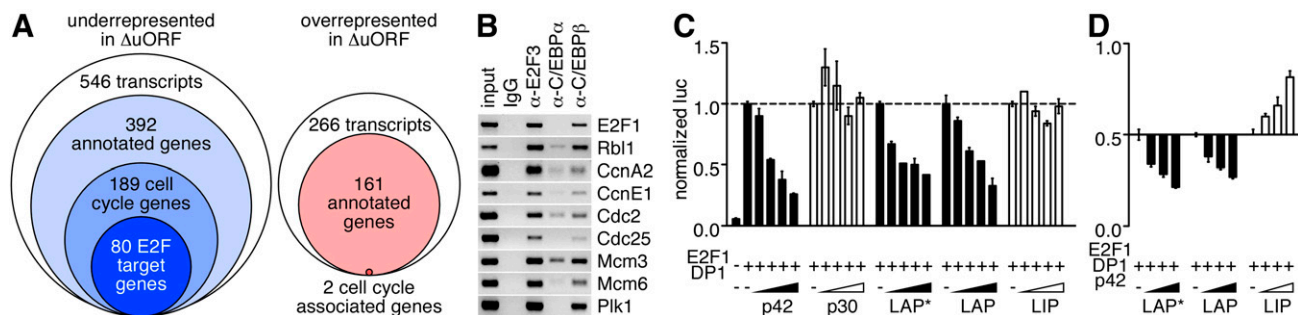

Figure 5. The C/EBPβ^{ΔuORF} mutation causes repression of E2F target genes. (A) Graphic representation of a genome-wide microarray expression analysis comparing transcript levels in C/EBPβ^{WT} and C/EBPβ^{ΔuORF} liver at 36 h after PH. (B) Representative ChIP assay on C/EBPβ^{WT} liver chromatin showing 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, intermediately repressive C/EBPα p42 expression (luciferase activity set to 0.5) showing the corepressive function of LAP* and LAP (black bars) and the derepressive 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 p30 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/EBPβ^{ΔuORF} livers and the rescue of this phenotype in C/EBPβ^{LIP} 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/EBPα and C/EBPβ isoforms to facilitate rapid cell cycle entry during liver regeneration.

The analysis of the C/EBPβ^{ΔuORF} mice proves the physiological relevance of uORF-mediated translational control in mammals. We note that low amounts of LIP can be detected in C/EBPβ^{ΔuORF} 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 thrombocythemia (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/EBPβ^{ΔuORF} and C/EBPβ^{WT} mice

Mutant (C/EBPβ^{ΔuORF}) and control (C/EBPβ^{WT}) mice were generated by homologous recombination according to standard protocols (Supplemental Fig. S2). The neomycin cassette was removed by crossing C/EBPβ^{ΔuORF} and C/EBPβ^{WT} 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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