Inactivation of CBF/NF-Y in postnatal liver causes hepatocellular degeneration, lipid deposition, and endoplasmic reticulum stress

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We previously demonstrated that CBF activity is needed for cell proliferation and early embryonic development. To examine the in vivo function of CBF in differentiated hepatocytes, we conditionally deleted CBF-B in hepatocytes after birth. Deletion of CBF-B resulted in progressive liver injury and severe hepatocellular degeneration 4 weeks after birth. Electron microscopic examination demonstrated pleiotropic changes of hepatocytes including enlarged cell and nuclear size, intracellular lipid deposition, disorganized endoplasmic reticulum, and mitochondrial abnormalities. Gene expression analyses showed that deletion of CBF-B activated expression of specific endoplasmic reticulum (ER) stress-regulated genes. Inactivation of CBF-B also inhibited expression of C/EBP alpha, an important transcription factor controlling various metabolic processes in adult hepatocytes. Altogether, our study reveals for the first time that CBF is a key transcription factor controlling ER function and metabolic processes in mature hepatocytes.

The endoplasmic reticulum (ER) stress pathway may play an important role in maintaining normal ER function and protecting hepatocytes from injury. A broad spectrum of insults to the liver, such as viral infections, metabolic disorders, and abuse of alcohol or drugs, can lead to ER stress1–3. Induction of a distinct ER stress pathway occurs in the livers of diabetic mice, as well as in mice fed high fat diets and genetic models of obesity4,5. While many studies have linked pathologic conditions of the liver with induction of ER stress, the factor(s) initiating activation of the ER stress pathway are unclear.

The ER stress pathway has evolved for cellular adaptation under various stress conditions such as increase in secretory protein synthesis, expression of misfolded proteins, glucose deprivation, perturbation in calcium homeostasis, and hypoxia. This pathway generally contains two major parts, 1) transcriptional stimulation of multiple chaperone genes to increase the protein-folding capacity of ER, and 2) general translational attenuation until normal ER function is restored. Prolonged ER stress can lead to cell death or various metabolic changes, including steatosis in liver6–8.

Previously, analysis of ER stress–regulated promoters identified a composite promoter element, ERSE, that binds several different transcription factor such as CBF/NF-Y (CBF), ATF6, and XBP-1, which mediate transcription activation during ER stress9,10. Among these transcription factors, CBF is constitutively expressed in mammalian cells, whereas both ATF6 and XBP-1 are activated by ER stress. CBF is needed for recruitment of ATF6 or XBP-1 to ERSE DNA, which then results in transcriptional activation of ER stress regulated genes11,12. Knockout of XBP-1 in mice resulted in liver abnormalities, suggesting that the ER stress pathway could control normal liver development13. Interestingly, multiple CBF binding sites are found in the promoters of various ER stress-regulated chaperone genes such as GRP78, ERP72, and protein disulfide isomerase11 that are needed for quality control of secretory proteins, suggesting that CBF may be essential for ER function in the liver under normal conditions.

Mammalian CBF consists of three subunits, CBF-A (NF-YB), CBF-B (NF-YA), and CBF-C (NF-YC), all of which are needed for DNA binding14. To understand the function of CBF in vivo, previously we utilized the gene-targeting method and Cre recombinase-loxP system to generate mouse strains harboring a conditional CBF-B
allele, B\textsuperscript{lox}, containing one loxP site in intron 2 and one loxP site in intron 8 of the CBF-B gene, and a B\textsuperscript{-} allele containing a deletion from exon 3 to exon 8 of the CBF-B gene\textsuperscript{15}. Heterozygous mice, with one B\textsuperscript{lox} and one B\textsuperscript{-} allele of CBF-B, were normal and fertile. However, no viable new-borns, and no embryos with homozygous B\textsuperscript{-} allele were ever obtained in the crosses between the heterozygous mice. These results indicated that the CBF activity is required for early embryonic development and viability.

We speculate that CBF may affect ER function in hepatocytes in the mature liver. To test this possibility, the mice harboring the conditional CBF-B allele, B\textsuperscript{lox}], were mated with Alb-Cre mice harboring a transgene containing cre recombinase under the control of albumin promoter/enhancer\textsuperscript{16}. The Alb-Cre mice express Cre recombinase and induce deletion of the genomic locus flanked by loxP sites specifically in hepatocytes. This resulted in deletion of CBF-B gene postnatally exclusively in liver. Inactivation of CBF-B caused severe liver injury with progressive degeneration of hepatocytes, and induction of an aberrant ER stress pathway. Our study revealed that CBF is needed for expression of a subset of ER stress-regulated protein disulfide isomerase genes as well as the C/EBP alpha transcription factor in postnatal hepatocytes.

### Results

**Conditional inactivation of CBF-B in liver of newborn mice.** To examine CBF-B deletion, 4-week old B\textsuperscript{lox}/Alb-Cre and B\textsuperscript{lox}/Alb-Cre mice were sacrificed to collect the liver, heart, and kidney for isolation of DNAs, which were used in PCR reactions to identify B\textsuperscript{lox} and B\textsuperscript{-} alleles. This showed that B\textsuperscript{-} allele was specifically generated in the liver but not in heart or kidney of B\textsuperscript{lox}/Alb-Cre mice, indicating that CBF-B gene was specifically deleted in liver (Fig. 1). Generation of the B\textsuperscript{-} allele was accompanied by reduction of B\textsuperscript{lox} allele in liver. The remaining B\textsuperscript{lox} allele in the liver indicated that recombination of loxP of CBF-B did not occur in every cell of the liver as described in a previous publication\textsuperscript{17}. Quantitative PCR was done to measure B\textsuperscript{lox} and B\textsuperscript{-} alleles in tissue DNAs and showed that approximately 60% of the B\textsuperscript{lox} allele was deleted in the liver of both B\textsuperscript{lox}/Alb-Cre and B\textsuperscript{lox}/Alb-Cre mice at 4 weeks of age (data not shown).

**Inactivation of CBF-B in liver resulted in progressive liver injury.** To test hepatocyte viability, blood samples of mice at 4 weeks of age were examined for alanine aminotransferase (ALT) and aspartate aminotransferase (AST). ALT and AST were elevated in the blood of knockout mice compared to control mice (Table 1). Total bilirubin was also increased in knockout mice. In contrast, both cholesterol and triglycerides were reduced, but creatinine concentration (a marker of kidney function) was not changed in knockout mice. These results indicated that the knockout mice were undergoing hepatocellular injury. When 12 B\textsuperscript{lox}/Alb-Cre and 12 B\textsuperscript{lox}/Alb-Cre mice were monitored after 4 weeks of age, 11 B\textsuperscript{lox}/Alb-Cre and 4 B\textsuperscript{lox}/Alb-Cre mice died between 4 and 6 weeks of age. In contrast, none of 41 littermate control mice died at this age (Table 2). These results indicated that the postnatal deletion of CBF-B in liver is lethal in mice. The higher death rate in B\textsuperscript{lox}/Alb-Cre line is likely due to a higher rate of CBF-B inactivation through a single CBF-B allele deletion, compared to

### Table 1 | Analysis of blood serum of mice at 4 weeks age a

| Genotype          | ALT (U/L) | AST (U/L) | Bilirubin (mg/dl) | Cholesterol (mg/dl) | Triglyceride (mg/dl) | Creatinine (mg/dl) |
|-------------------|-----------|-----------|-------------------|---------------------|---------------------|--------------------|
| **Knockout**      |           |           |                   |                     |                     |                    |
| B\textsuperscript{lox}/Alb-Cre | 341.00 ± 114.98 | 1528.67 ± 520.09 | 1.63 ± 0.40 | 57.11 ± 13.40 | 39.00 ± 9.89 | ND |
| B\textsuperscript{lox}/Alb-Cre | 291.50 ± 149.20 | 1206.75 ± 382.30 | 0.50 ± 0.14 | 70.50 ± 4.90 | 64.00 ± 2.83 | 0.27 ± 0.028 |
| **Control**       |           |           |                   |                     |                     |                    |
| B\textsuperscript{lox}/Alb-Cre | 46.20 ± 11.50 | 183.17 ± 74.13 | 0.33 ± 0.12 | 124.50 ± 11.80 | 93.00 ± 2.82 | 0.28 ± 0.014 |

*The data presented for each component is the mean and standard deviations for independent measurements of blood samples from 7 B\textsuperscript{lox}/Alb-Cre and 5 B\textsuperscript{lox}/Alb-Cre of knockout group, and 11 of control group mice. The standard deviations represented by error bars. The difference of ALT, AST, bilirubin, cholesterol, and triglycerides levels between the knockout and control group are significant (P<0.003), whereas the difference of creatinine level is not significant. The creatinine level of B\textsuperscript{lox}/Alb-Cre mice was not determined.

### Table 2 | Percent death of CBF-B knockout mice between 4 and 6 weeks of age

| Genotype          | Total mice | Died at 4-6 weeks | % Death |
|-------------------|------------|-------------------|---------|
| **Knockout**      |            |                   |         |
| B\textsuperscript{lox}/Alb-Cre | 12 | 11 | 91.7% |
| B\textsuperscript{lox}/Alb-Cre | 12 | 4 | 33.3% |
| **Control**       |            |                   |         |
| B\textsuperscript{lox}/Alb-Cre, B\textsuperscript{lox}/Alb-Cre, and B\textsuperscript{lox}/Alb-Cre | 41 | 0 | 0% |
Bflox/flox/Alb-Cre line, which needs two CBF-B alleles to be deleted for CBF-B inactivation.

The livers of knockout mice at 4 weeks of age were pale and nodular compared to littermate control mice (Fig. 2a–b). Histologic analysis at 2, 3, and 4 weeks revealed that the hepatocytes of knockout mice at 4 weeks were diffusely enlarged, as were their nuclei, and contained many microvacuoles within the cytoplasm (Fig. 2f) compared to the hepatocytes of controls at the same age (Fig. 2e). In addition, the livers of knockout mice displayed focal hepatocyte necrosis, mild lobular inflammation, increased numbers of sinusoidal cells, focal hyperplasia of bile ducts, and multiple regenerative nodules (Fig. 2g).

At 2 weeks age, the enlargement of hepatocytes was already evident in the knockout liver, but other changes in hepatocytes were less significant (Fig. 2d). By 3 weeks age, in addition to enlargement of hepatocytes, mild degenerative changes in hepatocytes including occasional necrotic and apoptotic cells were also observed in the knockout liver (Fig. 2h). Together, this analysis indicated that a progressive injury of the hepatocytes was due to the deletion of CBF-B gene after birth.

The sections of frozen liver tissue at 4 weeks of age stained with Oil Red O (ORO) showed that a large amount fat in the livers of knockout mice (Fig. 3). In contrast, no fat deposition was observed in the livers of control mice. Electron microscopy at 4 weeks age showed that the hepatocytes of knockout mice contained many lipid droplets, which accumulated in both cytosol and nucleus (Fig. 4b), and also displayed dilated smooth endoplasmic reticulum, and total depletion of glycogen (Fig. 4d). In addition, mitochondria displayed pleomorphism in the knockout hepatocytes compared to uniformity in control hepatocytes; additionally some mitochondria were elongated or circular or contained occasional paracrystalline arrays, none of which were observed in controls.

Figure 2 | Knockout of CBF-B results in hepatomegaly and liver steatosis. (a) Liver morphology of wild type and knockout mice. Photo shows livers from WT (left) and knockout mouse (right). The knockout livers had rough, pale to nearly white surface. (b) Histological analysis of liver sections from 4 weeks WT and knockout mice, (H&E stain, x12). (c–f) Hepatocellular injury after knockout of CBF-B at 2 and 4 weeks after birth, compared to wild type controls. The hepatocytes at 2 weeks are already larger than controls and their nuclei are also enlarged (upper panel). At 4 weeks, the hepatocytes of the control have grown but those of the knockout mouse are much larger with much larger and pleomorphic nuclei. They also contain numerous cytoplasmic vacuoles consistent with steatosis, and there are scattered small foci of hepatocellular necrosis and accompanying mid intralobular inflammation, (H&E stain, x400). (g) Livers of knockout mice at 4 weeks display focal bile ductular hyperplasia (arrowheads), and focal regenerative nodules (arrow), (H&E, x100). h, Arrows point to individual cell necrosis in 3 weeks CBF-B knockout livers. Cells are enlarged, have pale homogeneous cytoplasm and either a shrunken nucleus (upper left) or no apparent nucleus, (H&E, X200).

Figure 3 | Accumulation of fat in liver after knockout of CBF-B gene at 4 weeks. Frozen liver sections were stained with Oil red O, (x400). Significant lipid deposition was observed in knockout livers (right).

Figure 4 | Electron microscopy of hepatocytes after knockout of CBF-B gene, compared with controls at age of 4 weeks. Arrowheads in b panel indicate lipid droplets in knockout hepatocytes (x10,000). An arrowhead in c panel (x25,000) indicates normal abundance of glycogen rosettes in control hepatocytes, whereas none are found in the knockouts. The long arrow in d panel indicates an abnormal mitochondrion with a pseudocrystalline inclusion in knockout hepatocytes; other mitochondria in the figure display variation in size and shape. The short arrow in d panel indicates dilated smooth endoplasmic reticulum in knockout hepatocytes.
Inactivation of CBF-B resulted in altered expression of endoplasmic reticulum stress regulated genes. Based on previous promoter studies, we hypothesized that the loss of CBF activity could cause inhibition in expression of genes that are regulated during the cell cycle or during endoplasmic reticulum (ER) stress. To test this possibility, we isolated total RNA from livers of both control and knockout mice at 1, 2, 3, and 4 weeks of age after birth. Initially, we examined expression of XBP-1(s) and GADD153/CHOP (CHOP) for ER stress, cyclin B1 for cell cycle, and CBF-B to check for knockout efficiency. The XBP-1(s) mRNA, which is generated by splicing of XBP-1(u) mRNA as a result of IRE-1 kinase/endoronuclease activation, was measured by RT-PCR (Fig. 5a).

Expression of CBF-B, CHOP, and cyclin B1 mRNAs was measured by quantitative RT-PCR (QRT-PCR) (Fig. 5b). Expression of both XBP-1(s) and CHOP was significantly activated in knockout but not in control livers at 1, 2, and 3 weeks, and modestly activated at 4 weeks of age. In contrast, expression of cyclin B1 was very similar between control and knockout mice, with a small reduction in liver of 4 weeks age knockout mice. This indicated that loss of CBF activity in liver specifically resulted in sustained activation of ER stress pathway from 1 week to 3 weeks, and continued at a modest level at 4 weeks after birth when significant pathology was observed. We then examined expression of several other genes that are also reported to be activated during ER stress. This demonstrated that loss of CBF activity also resulted in activation of expression of GRP78 in knockout livers at 2 and 3 weeks ages, but a slight reduction at 4 weeks age (table 3). In contrast, loss of CBF activity resulted in reduction of expression of three protein disulfide isomerase (PDI) family genes, ERP72, PDI and PDIA3, with a progressive reduction from 2 to 4 weeks. For example, expression of ERP72 was reduced by 9-fold at 4 weeks compared to 1.9-fold and 2.7-fold at 2 and 3 weeks. We also measured expression of C/EBP family and PPAR gamma transcription factors genes, which reflect the metabolic state of the liver17,18. Expression of C/EBP alpha but not C/EBP beta was significantly reduced in the knockout livers at 2, 3, and 4 weeks of age (table 3). In contrast, expression of C/EBP delta and PPAR gamma was slightly increased in the knockout livers. To further confirm that ER stress induction is one of initial event after loss of CBF-B activity, we did gene expression analysis by northern blot after birth and at 1 week age. The expression of GRP78 and ERP72 dramatically changed just after birth (Fig. 5c), which suggested that ER stress is induced at early stage. Together, this analysis indicated that expression of PDI family and C/EBP alpha genes are dependent on CBF activity in postnatal liver.

Discussion

Our study demonstrates that deletion of CBF-B gene in liver resulted a progressive injury to hepatocytes, with significant changes in both cell and nuclear size, and other changes including focal necrosis, bile duct proliferation, and development of regenerative nodules at 4 weeks after birth of mice. Electron microscopy showed that inactivation of CBF-B resulted in multiple alterations of hepatocytes, which include intracellular and intranuclear lipid deposition, dilation of endoplasmic reticulum, mild pleomorphism of mitochondria, and marked depletion of glycogen. The endoplasmic reticulum became dilated in CBF-B knockout livers in response to the elevation of ER chaperone GRP78. This observation is consistent with previous finding19. Deposition of lipids in hepatocytes lacking CBF-B was also confirmed by Oil red O staining. Altogether, our study demonstrates that CBF-B, and thus CBF activity is essential for normal metabolic homeostasis of hepatocytes in vivo in mice after birth.

The gene expression analysis showed that deletion of CBF-B resulted in generation of XBP-1(s), and also stimulation of CHOP and GRP78 gene expression, all of which are usually activated by the unfolded protein response signaling pathway due to ER stress6. The inactivation of CBF, however, did not change expression of a cell cycle regulator, cyclin B1, which was found to be dependent on CBF in cultured cells in vitro20. This indicated that CBF is specifically needed for ER function in hepatocytes. Further analysis in expression of other ER stress regulated genes showed that inactivation of CBF inhibited expression of ERP72, PDI, and PDIA3, which are members of protein disulfide isomerase (PDI) family that play role in oxidative protein folding. Thus, expression of ERP72, PDI, and PDIA3 is dependent on CBF activity in hepatocytes in vivo. A recent study demonstrated that inhibition of PDI activity induced ER stress and activated unfolded protein response in neuronal cells21; however no such study has been done in hepatocytes. We speculate that the inhibition in expression of the PDI family genes due to loss of CBF
Table 3: Analysis of expression of different genes in total RNAs of control and knockout (KO) mice liver.

| Gene | Control 4 weeks | Knockout 4 weeks | Fold | Control 3 weeks | Knockout 3 weeks | Fold | Control 2 weeks | Knockout 2 weeks | Fold |
|------|-----------------|------------------|------|-----------------|------------------|------|-----------------|------------------|------|
| ERP72 | 0.00078 | 0.046 | 5.66 | 0.00072 | 0.046 | 5.66 | 0.032 | 0.017 | 1.88 |
| PDI  | 0.095 | 1.82 | 19.38 | 0.151 | 0.59 | 3.88 | 0.058 | 0.341 | 5.88 |
| Pdia3 | 0.0404 | 0.597 | 14.88 | 0.0556 | 0.603 | 10.98 | 0.023 | 0.0112 | 2.08 |
| C/EBP | 0.0143 | 0.00455 | 3.07 | 0.00115 | 0.00603 | 5.27 | 0.00738 | 0.0275 | 3.67 |
| bC/EBP | 0.00486 | 0.129 | 2.67 | 0.000538 | 0.00815 | 1.48 | 0.000839 | 0.0112 | 1.38 |
| dC/EBP | 0.00329 | 0.000226 | 14.88 | 0.000538 | 0.00815 | 1.48 | 0.000839 | 0.0112 | 1.38 |

**Methods**

**Animals.** The generation of Bflox/+ and Bflox/+ mice maintained on a C57BL6 background has been described previously. The Alb-Cre transgenic mice harboring a transgene containing Cre recombinase under control of an albumin enhancer/promoter.
promoter, were obtained from the Jackson Laboratory (strain name: B6.Cg-Tg(Alb-Cre)21Mgn/J), and were maintained as hemizygotes. The Bflox/flox mice were bred with the Alb-Cre mice (homozygous for wild type CBF-B, Bflox/flox) to generate Bflox/flox/Alb-Cre mice (one wild type and one null CBF-B allele; hemizygous for Alb-Cre). These were then mated with Alb-Cre mice to generate Bflox/flox/Alb-Cre and Bflox/Cre/Alb-Cre mice, which were used as knockout mice to examine the effects of CBF-B deletion in the liver. The littersmates Bflox/flox/Alb-Cre, Bflox/Cre/Alb-Cre, Bflox/+, and Bflox/Cre were used as controls for each subsequent experiment. The mice were housed at the University of Texas M. D. Anderson Cancer Center animal facility according to the National Institutes of Health guidelines on the use of laboratory and experimental animals. All animal procedures are carried out as per the institutional guidelines for use of laboratory animals and as approved by the Ethics Committee of MDACC. Mice were given ad libitum access to food and water and underwent no treatment. Animals were euthanized at given time points, and the livers and other organs were quickly removed for various tissue preparations.

**Serum analysis.** Blood from 4-week old mice was collected by tail-vein bleeding in BD Microtainer Serum Separator Tubes. Serum was separated by high-speed centrifugation of the blood at 14,000 rpm for 2 minutes at room temperature. The serum samples were then assayed for aspartate aminotransferase (AST), alanine transaminase (ALT), bilirubin, cholesterol, and creatinine levels using COBAS 7170 automatic analyzer.

**Histology.** Liver specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 microns and stained with hematoxylin and eosin. Hematoxylin and eosin stained slides were examined with a Leica DM 2500 microscope using 10 × 0.40, 20 × 0.70 HC, and 40 × 0.85 HCX plan APO Leica objectives (Leica Microsystems, Banockburn, Illinois). A Spot Insight 18.2 Color Mosaic camera (Diagnostic Instruments, Sterling Heights, Michigan) was used to acquire photomicrographs. Cell and nuclear measurements of hepatocytes were obtained with Spot software PC version 4.6.7.4 (Diagnostic Instruments, Sterling Heights, Michigan).

**Electron Microscopy.** 0.2 cm cubic portions of liver were immersed in 3% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.4. After overnight fixation they were transferred to 0.1 mol/L phosphate buffer and then postfixed in 1% OsO4 for 2 hours. The tissue was dehydrated in ethanol, rinsed in propylene oxide, and embedded in Epon. Slabs (400 μm) were cut on a Reichert ultramicrotome, stained with uranyl acetate, and viewed and photographed in a JOEL 101CX transmission electron microscope.

**Oil Red O Staining.** Freshly dissected liver sections were embedded in OCT and snap frozen in isopentane to a temperature of −80°C. Frozen sections at 6–7 microns were stained in a 0.3% solution of Oil Red O in 60% isopropanol for 1 h. After washing in 60% isopropanol, sections were counterstained with Gills hematoxylin. Sections were examined under bright-field microscopy with an Olympus model BX50 photomicroscope.

**Extraction of RNA, Real-Time RT-PCR, and Northern Blot.** Total RNA was extracted from liver at various time points after birth from both control and knockout mice (a minimum of 3 mice per group was used for each group at each time point) using an RNeasy mini kit (QIAGEN). Real-time RT-PCR was performed using the ABI PRISM 7200 (Applied Biosystems), and the results were analyzed with the 7170 automatic analyzer.

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