Hepatocyte Nuclear Factor 4α Is Implicated in Endoplasmic Reticulum Stress–Induced Acute Phase Response by Regulating Expression of Cyclic Adenosine Monophosphate Responsive Element Binding Protein H

Jennifer Luebke-Wheeler,[1] Kezhong Zhang,[2] Michele Battle,[1] Karim Si-Tayeb,[1] Wendy Garrison,[1] Sodhi Chhinder,[1] Jixuan Li,[1] Randal J. Kaufman,[2-4] and Stephen A. Duncan[1]

Loss of the nuclear hormone receptor hepatocyte nuclear factor 4α (HNF4α) in hepatocytes results in a complex pleiotropic phenotype that includes a block in hepatocyte differentiation and a severe disruption to liver function. Recent analyses have shown that hepatic gene expression is severely affected by the absence of HNF4α, with expression of 567 genes reduced by ≥2.5-fold ($P \leq 0.05$) in *Hnf4α*$^{-/-}$ fetal livers. Although many of these genes are direct targets, HNF4α has also been shown to regulate expression of other liver transcription factors, and this raises the possibility that the dependence on HNF4α for normal expression of some genes may be indirect. We postulated that the identification of transcription factors whose expression is regulated by HNF4α might reveal roles for HNF4α in controlling hepatic functions that were not previously appreciated. Here we identify cyclic adenosine monophosphate responsive element binding protein H (*CrebH*) as a transcription factor whose messenger RNA can be identified in both the embryonic mouse liver and adult mouse liver and whose expression is dependent on HNF4α. Analyses of genomic DNA revealed an HNF4α binding site upstream of the CrebH coding sequence that was occupied by HNF4α in fetal livers and facilitated transcriptional activation of a reporter gene in transient transfection analyses. Although CrebH is highly expressed during hepatogenesis, *CrebH*$^{-/-}$ mice were viable and healthy and displayed no overt defects in liver formation. However, upon treatment with tunicamycin, which induces an endoplasmic reticulum (ER)–stress response, *CrebH*$^{-/-}$ mice displayed reduced expression of acute phase response proteins. *Conclusion:* These data implicate HNF4α in having a role in controlling the acute phase response of the liver induced by ER stress by regulating expression of CrebH. (HEPATOLOGY 2008;48: 1242-1250.)

Abbreviations: Alb1, albumin 1; Apoc3, apolipoprotein c3; ATF, activating transcription factor; ChIP, chromatin immunoprecipitation; Creb, cyclic adenosine monophosphate responsive element binding protein; CRP, C-reactive protein; DE, definitive endoderm; EMSA, electrophoretic mobility shift analysis; ER, endoplasmic reticulum; Fox, forkhead box; H&E, hematoxylin-eosin; HIV, human immunodeficiency virus; HNF, hepatocyte nuclear factor; mRNA, messenger RNA; PCR, polymerase chain reaction; RT-PCR, reverse-transcription polymerase chain reaction; SAA3, serum amyloid A3; SAP, serum amyloid P-component; Ttr, transthyretin; VE, visceral endoderm.

From the [1]Department of Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, Milwaukee, WI; and [2]Department of Biological Chemistry, [3]Department of Internal Medicine, and [4]Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, MI.

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Address reprint requests to: Stephen A. Duncan, Department of Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, Milwaukee, WI 53226. E-mail: duncans@mcw.edu.

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Hepatocyte gene expression is controlled by a complex network of transcription factors that is established during hepatogenesis.[1,2] Recent studies have shown that the nuclear hormone receptor hepatocyte nuclear factor 4α (HNF4α), as part of this network, is essential for initiating and maintaining hepatocyte differentiation and liver function.[3-6] Loss of HNF4α in the fetal hepatoblasts results in reduced expression of over 500 genes encoding factors that contribute to all aspects of hepatic function.[6] HNF4α has also been shown to regulate expression of other transcription factors, including HNF1α[7,8] and peroxisome proliferator-activated receptor α,[9] and this suggests that regulation of hepatocyte gene expression by HNF4α may in some cases be indirect. We therefore sought to uncover novel transcription factors that were expressed during hepatic development that could potentially be regulated by HNF4α. We proposed that identification of such factors would implicate HNF4α in regulating aspects of liver function that heretofore were not recognized. In the current study, we report the identification of a cyclic adenosine monophosphate responsive element binding protein H (CrebH) (encoded by the gene *Creb3l3*), as a new target of HNF4α regulation that is expressed in differentiating hepatocytes throughout liver development and is essential for the expression of acute phase response proteins induced by endoplasmic reticulum (ER) stress.

Materials and Methods

Oligonucleotide Array Analysis. RNA was isolated from tissues with the RNeasy kit (Qiagen), and complementary RNA probes were prepared according to the directions described in the Affymetrix expression analysis technical manual.

RT-PCR. RT-PCR[3] and quantitative real-time RT-PCR[20] were performed as described previously. Primer sequences are available upon request.

In Situ *Hybridization and Immunohistochemistry.* *In situ* hybridization was performed as described elsewhere.[23] A probe used to detect CrebH was generated by *in vitro* transcription of a fragment from *CrebH* complementary DNA, which was generated by PCR using primers atcgaattcccatcagccctttcaactcc and atcggatccaggccagcctggtctacaag and subsequently cloned into the *Eco*R1/*Bam*H1 sites of pBSII-KS. Immunohistochemistry was performed with previously described procedures[5] with antibodies that detect HNF4α (Santa Cruz sc-6556;1:500).

Chromatin Immunoprecipitation and EMSA. Immunoprecipitation of chromatin was performed with the Upstate ChIP assay kit (Upstate #17-295) as described previously,[6] and EMSA was performed as described elsewhere.[17]

Analysis of Luciferase Expression. DNA was introduced into 293T cells with Lipofectamine Plus reagent (Invitrogen). Cell extracts were collected 48 hours after transfection and processed with a luciferase assay dual reporter system (Promega). Each experiment was performed in triplicate, the entire experiment was repeated on five separate occasions, and data were combined. Statistical significance was determined with the Student *t* test, with $P \leq 0.05$ considered significant.

Animals and Embryonic Stem Cells. The Medical College of Wisconsin or the University of Michigan Medical Center Institutional Animal Care and Use Committee approved all animal experiments and procedures. The generation of *Hnf4α*$^{+/-}$ (*Hnf4α*^{tm1Dnl}), *Hnf4α*$^{loxP/loxP}$ (*Hnf4α*^{tm1Sad}), *Foxa3Cre* [*Tg*(*Foxa3-cre*)^{1Khk}], *AlfpCre* [*Tg(Alb1-cre)*^{1Khk}], and *VillinCre* [*Tg(Vil-cre)*^{997Gum}] mice has been described previously.[5,16,17,24-26] Noon on the day of the appearance of a vaginal plug was considered to be 0.5 days post coitum, and the genotype of all embryos was determined by PCR analysis of genomic DNA. Pregnant mare serum gonadotropin, used in superovulation, was obtained from A.F. Parlow at the National Hormone and Peptide Program.

Results

CrebH Messenger RNA (mRNA) Is Highly Enriched in the Fetal Liver. Using Affymetrix oligonucleotide array analyses, we attempted to identify novel transcription factors whose expression was enriched in fetal livers. RNA was isolated from pools of livers, hearts, and heads that were dissected from E10.5 mouse embryos and used to generate probes that were hybridized to Affymetrix oligonucleotide GeneChip Murine Genome U74v2 A and B arrays. A comparison of liver, heart, and head arrays revealed 1208 genes whose expression was predicted to be increased >3.0-fold in the fetal liver samples compared to the heart and head samples. Of these genes, 78 encoded potential fetal liver–enriched transcription factors.[10] This list included Hnf4α, CCAAT/enhancer binding protein alpha, Forkhead box A1 (Foxa1), and Foxa3 (see Supplementary Table 1), all which have previously been shown to be expressed in hepatoblasts during embryonic development; however, other hepatoblast transcription factors, including HNF1α, HNF1β, and HNF6, were not identified, which suggested that the screen was not saturated.

Of the mRNAs identified, we chose to focus our analyses on *CrebH* because it was highly enriched in the fetal

liver and was a member of the ATF/CREB family, other members of which are known to have important roles in controlling liver gene expression, and analyses of the human homolog of CrebH (CREBH) had been described as being exclusively expressed in the adult liver and undetectable in other tissues.[11] Using reverse-transcription polymerase chain reaction (RT-PCR), we first confirmed that *CrebH* mRNA was differentially expressed in the liver compared to the heart and head. Figure 1A shows that, like *Albumin 1* (*Alb1*) mRNA, *CrebH* mRNA was enriched in three independent E10.5 liver samples compared to heart and head samples. Real-time quantitative RT-PCR (not shown) demonstrated that *CrebH* mRNA levels were 45 times greater in the liver compared to the heart.

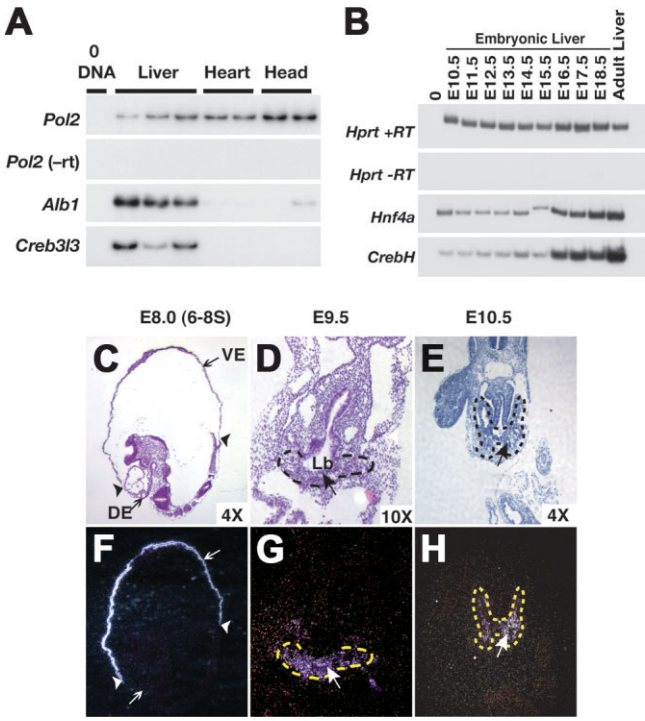
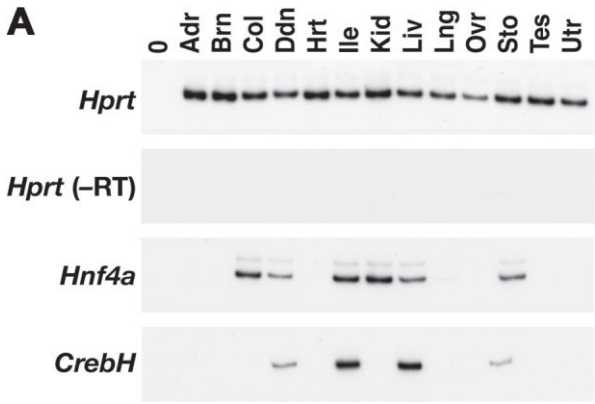


Fig. 1. Fetal expression of CrebH initiates in the primary liver bud and continues throughout hepatogenesis. (A) RT-PCR analyses revealed the presence of *CrebH* and *Alb1* mRNAs in livers isolated from E10.5 embryos. Amplification of *Rna pol2* (*Pol2*) was used as a loading control, whereas reactions lacking reverse transcriptase (−RT) and a DNA template (0DNA) confirmed the absence of contaminating DNA. (B) RT-PCR analyses uncovered *CrebH* and *Hnf4α* mRNA in livers isolated from mouse embryos at daily intervals ranging from E10.5 to E18.5 and in adult livers. Amplification of hypoxanthine guanine phosphoribosyl transferase (*Hprt*) was used as a loading control, whereas reactions lacking reverse transcriptase (−RT) and a DNA template (0DNA) confirmed the absence of contaminating DNA. (C-H) Radioactive *in situ* hybridization analyses revealed the presence of *CrebH* mRNA (arrows; silver grains) during early development. (C,F) Sagittal sections through an E8.5 (6-8 somite stage) embryo identified *CrebH* mRNA in the extraembryonic visceral endoderm (VE) but not in the definitive endoderm (DE); the extraembryonic/embryonic boundary is indicated by arrowheads. *CrebH* mRNA was also found to be present (D,G) in the primary liver bud (Lb; outlined with dashes) in transverse sections through an E9.5 embryo and (E,H) in the expanding clusters of hepatoblasts (outlined with dashes) in transverse sections through an E10.5 embryo. (C,D,E) H&E-stained bright-field images and (F,G,H) corresponding dark-field images are presented.



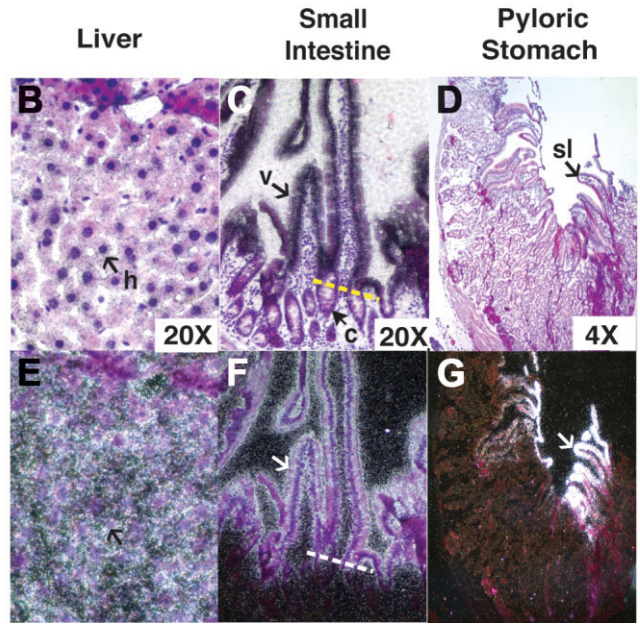


Fig. 2. CrebH is expressed in the adult liver and gastrointestinal tract. (A) RT-PCR analyses of *CrebH* and *Hnf4α* were performed on mRNA extracts from the adrenal gland (Adr), brain (Brn), colon (Col), duodenum (Ddn), heart (Hrt) ileum (Ile), kidney (Kid), liver (Liv), lung (Lng), ovary (Ovr), stomach (Sto), testis (Tes), and uterus (Utr). (B-G) The distribution of *CrebH* mRNA in (B,E) the liver, (C,F) small intestine, and (D,G) stomach was identified (white arrows, silver grains) with radioactive *in situ* hybridization analysis. *CrebH* mRNA was present in the hepatocytes (h, arrow) of the liver, in the epithelial cells of the villi (v, arrow) but not the crypts (c, white arrows; a yellow dashed line demarcates the villi/crypt border) of the small intestine, and in the surface lining cells (sl) of the stomach. (B-D) H&E-stained bright-field images and (E-G) corresponding dark-field images are presented.

Hepatic Expression of* CrebH *Initiates During Liver Bud Formation and Continues Throughout Hepatogenesis. As a first step toward determining whether CrebH had the potential to act downstream of HNF4α during liver development and function, we com-

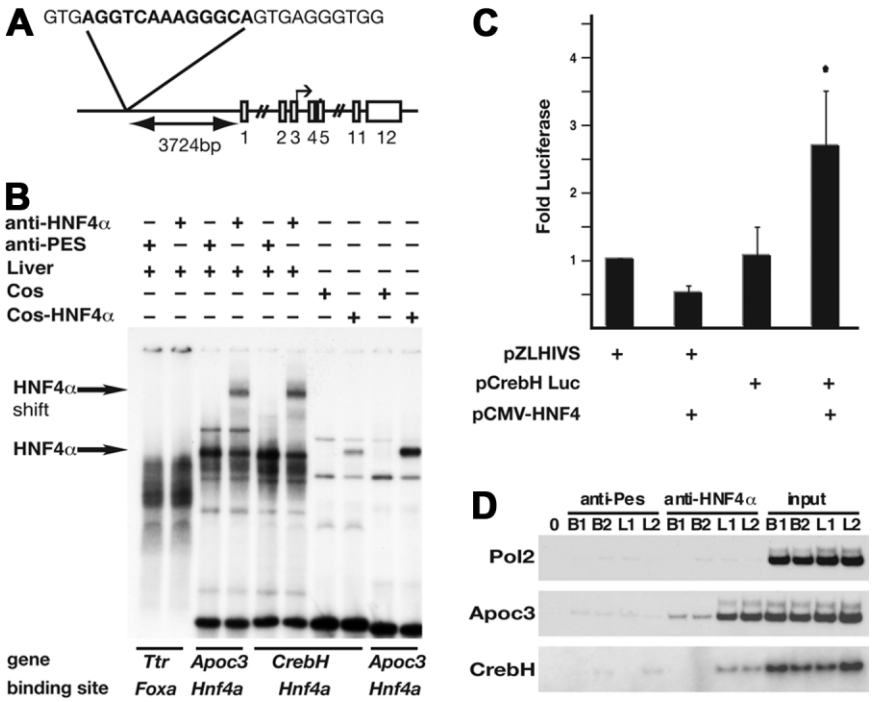


Fig. 3. *CrebH* is a direct target of HNF4α transcriptional activity. (A) Schematic showing the genomic location and sequence of the identified HNF4α binding site with respect to *CrebH* (exons are shown as boxes). (B) The ability of the HNF4α protein to bind the putative HNF4α-binding site was confirmed by EMSA. Radiolabeled oligonucleotides representing binding sites were incubated with liver nuclear extracts in the presence of anti-HNF4α antibody, and this resulted in a retarded migration of HNF4α-bound complexes (arrows) or anti-Pes1 antibody (negative control). Alternatively, EMSAs were performed with nuclear extracts from COS-7 cells or COS-7 cells expressing HNF4α. A previously described HNF4α binding site in the *Apoc3* promoter served as a positive control, and a *Foxa* (*Hnf3*) binding site within the *Ttr* promoter served as a negative control. (C) 293T cells were transfected with plasmids in which expression of luciferase was driven by the HIV basal promoter (pZLHIVS) or, in addition, a 207-bp fragment from the *CrebH* gene that contained the HNF4α binding site (pCrebH-Luc1) in the presence or absence of exogenously expressed HNF4α. Luciferase levels from five independent experiments are presented as fold differences with respect to cells transfected with pZLHIVS alone. Significance was determined by the Student *t* test ($P < 0.05$). (D) ChIP analyses were performed on chromatin extracted from two independent E18.5 livers (L1 and L2) or brains (B1 and B2), which acted as negative control tissue that did not express HNF4α. Chromatin was precipitated with anti-HNF4α or anti-Pes1 (negative control), and specific primers were used to amplify input chromatin or chromatin precipitated from the *Pol2* promoter (negative control), *Apoc3* promoter (positive control), or *CrebH*.

pared expression of *CrebH* to that of *Hnf4α* during hepatic development. Livers were isolated from embryos at E10.5 through E18.5 and from adults, and mRNAs encoding CrebH and HNF4α were measured by RT-PCR. Figure 1B shows that *CrebH* is expressed in the liver at low levels early in development, begins to increase around E14.5, and continues increasing until maximal expression is reached in the adult liver. This dynamic pattern of expression is very similar to that of *Hnf4α*, which was also seen to increase over this developmental time course. *In situ* hybridization analyses were next performed to assess expression of *CrebH* mRNA during the onset of hepatic development. Figure 1C,F shows that during specification of the hepatic lineage from the ventral endoderm at E8.0 (6-8 somites), *CrebH* mRNA is restricted to the extraembryonic visceral endoderm, and it was not detected in the definitive endoderm, as has previously been described for *Hnf4α*.[12,13] Approximately a day later in development at E9.5, like *Hnf4α*,[12,13] *CrebH* mRNA was detected in the definitive endoderm forming the primary liver bud (Fig. 2D,G), and expression continued in the hepatoblasts as they delaminated from the bud and invaded the surrounding septum transversum at E10.5 (Fig. 1E,H).

CrebH *Is Expressed in the Epithelial Cells of the Adult Liver, Pyloric Stomach, and Small Intestine.* The expression profile of *CrebH* mRNA in adult mouse tissues was next compared to that of *Hnf4α*. RT-PCR analysis was performed with complementary DNA derived from a spectrum of adult mouse tissues (Fig. 2A). *Hnf4α* mRNA was identified in a variety of adult epithelial tissues, including the liver, kidney, and gastrointestinal tract, as described previously.[13-15] Like *Hnf4α*, *CrebH* mRNA was identified in the liver, pyloric stomach, duodenum, and ileum; however, in contrast to *Hnf4α*, *CrebH* mRNA was not identified in the kidney or colon. In sum, all tissues that expressed *CrebH* also expressed *Hnf4α* mRNA, although not all *Hnf4α*-positive tissues expressed *CrebH*.

We next performed *in situ* hybridization to identify the specific cell types that expressed *CrebH* mRNA. Tissues lacking *CrebH* mRNA, such as the kidney (based on RT-PCR analysis), showed no hybridization above background and therefore acted as convenient negative controls (data not shown). *CrebH* mRNA was detected in the hepatocytes of the adult liver (Fig. 2B,E) and in the epithelium of the villi, but not the crypts, of the small intestine (Fig. 2C,F). *CrebH* transcripts were also identified in the surface epithelial cells of the pyloric stomach (Fig. 2D,G) but not in the glands.

HNF4α Regulates Transcription Through an HNF4α-Binding Site Within the Putative Transcriptional Regulatory Region of the* CrebH *Gene. The aforementioned expression studies demonstrated that following the specification of the hepatic cell lineage, CrebH is continuously expressed in the hepatic cells throughout liver development in a manner indistinguishable from HNF4α, raising the possibility that *CrebH* is a direct transcriptional target of HNF4α. To identify potential DNA sequences that could facilitate HNF4α-mediated expression of *CrebH*, we therefore examined 27.5 kb of the *CrebH* genomic DNA sequence, including sequences extending 10 kb upstream of exon 1, for the presence of any of 215 known HNF4α binding sites, using an HNF4α motif finder generated by Sladek and colleagues (http://www.sladeklab.ucr.edu/links.html). This analysis identified a single potential HNF4α binding site (H4.77) lying 3.7 kb upstream of *CrebH* exon 1 (Fig. 3A). The ability of HNF4α to bind the aforementioned sequence was confirmed with electrophoretic mobility shift analyses (EMSAs) on nuclear extracts from adult liver. Figure 3B shows that HNF4α protein could be detected in a complex with a well-characterized binding site (H4.21; see http://bioinfo.ucr.edu/~ebolotin/h4supp.html) from the *Apolipoprotein c3* (*Apoc3*) gene,[14] which could be converted to a slower migrating complex by inclusion of anti-HNF4α antibody. A complex with a similar migration pattern was identified when the same extracts were incubated with the H4.77 HNF4α-binding site from the *CrebH* gene, but not when extracts were incubated with a FoxA transcription factor binding site from the *Transthyretin* (*Ttr*) gene, which acted as a control for binding specificity. Similar results were obtained when extracts from COS-7 cells expressing exogenous HNF4α were used (Fig. 3B). Other protein-DNA complexes were also detected in the extracts and likely reflect the binding of additional proteins known to interact with HNF4α binding sites, such as retinoid A receptor, retinoid X receptor, and chicken ovalbumin upstream promoter transcription factor.

The ability of exogenous HNF4α to activate transcription via the HNF4α binding site within the putative *CrebH* promoter region was studied by transient transfection analyses in 293T cells, which do not express endogenous HNF4α. Figure 3C shows that luciferase levels measured in 293T cells transfected with a reporter plasmid containing only the human immunodeficiency virus (HIV) basal promoter to regulate transcription of the luciferase reporter gene (pHIV-Luc) were not affected by the additional introduction of exogenously expressed HNF4α. Similar results were obtained when 293T cells were transfected with the same reporter plasmid containing an additional 123-bp element from *CrebH* that included the HNF4α-binding site (pCrebH-Luc). However, the introduction of exogenously expressed HNF4α to pCrebH-Luc–transfected 293T cells now resulted in an approximately 2.5-fold induction (Student *t* test, $P \leq 0.05$) of luciferase activity in comparison with controls, and this demonstrated that HNF4α can activate transcription through this element of the *CrebH* gene.

Using chromatin immunoprecipitation (ChIP) analyses, we next addressed whether HNF4α could occupy the identified binding site within the endogenous *CrebH* gene in fetal livers. Figure 3D shows that, in contrast to se-

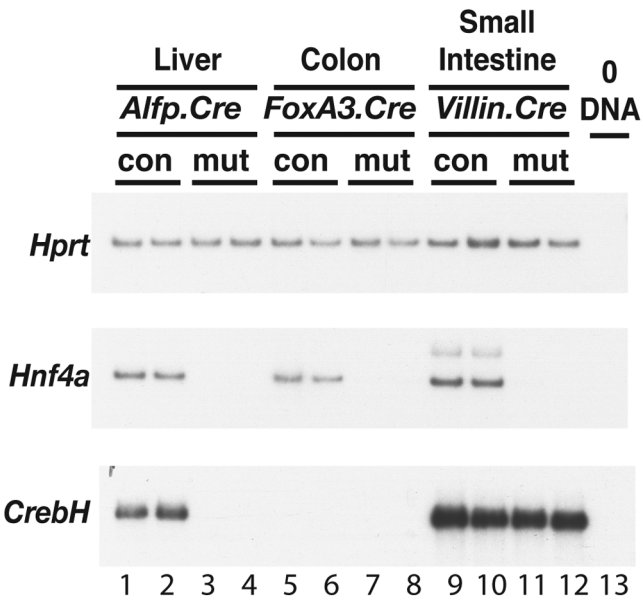


Fig. 4. HNF4α is essential for expression of CrebH in the liver but is dispensable for expression in the small intestine. RT-PCR analyses of *Hnf4α* and *CrebH* mRNA were performed on RNA isolated from liver (lanes 1-4), colon (lanes 5-8), or small intestine (lanes 9-13) that had been collected from $Hnf4\alpha^{loxP/+}$ (con) or $Hnf4\alpha^{loxP/loxP}$ (mut) mice that expressed Cre recombinase in the hepatocytes (*Alfp.Cre*; lanes 1-4), colonic epithelial cells (*Foxa3.Cre*; lanes 5-8), or small intestine epithelial cells (*Villin.Cre*; lanes 9-13), respectively. Amplification of hypoxanthine guanine phosphoribosyl transferase (*Hprt*) was used as a loading control, and omitting DNA template from the reaction (0DNA) served as a negative control.

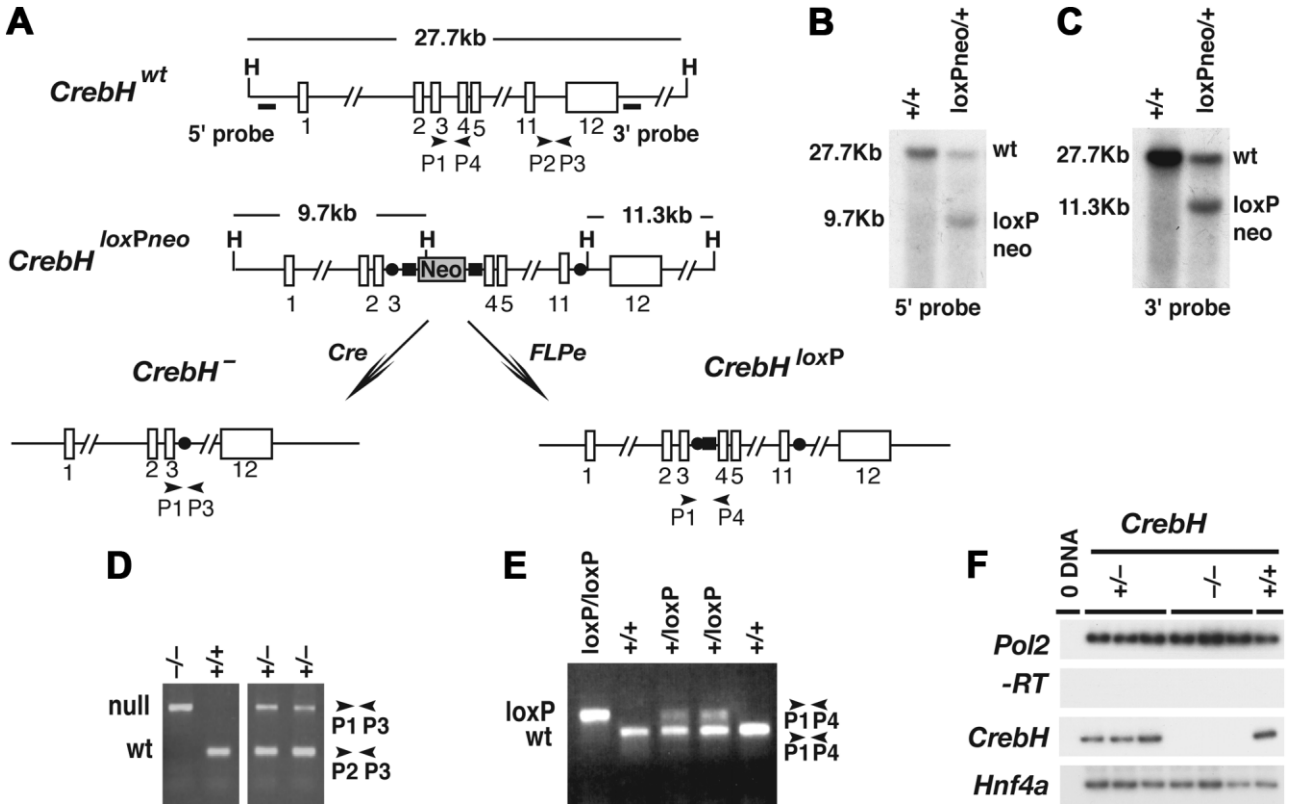


Fig. 5. Generation of mice harboring conditional and null alleles of *CrebH*. (A) Diagram showing the targeting strategy used to generate a *CrebH*^{loxPneo} allele as well as the alleles CrebH^{-} (in response to Cre recombinase activity) and *CrebH*^{loxP} (in response to Flpe recombinase activity). The position of *HindIII* restriction endonuclease recognition sequences (H), a *neomycin phosphotransferase* cassette (Neo), *lox*P (solid circles) and *Frt* (solid rectangles) sites, oligonucleotide PCR primers, and Southern blot probes are shown with respect to exons (numbered open rectangles). (B,C) Autoradiograph of a Southern blot of *HindIII*-digested embryonic stem cell genomic DNA hybridized to (B) 5′ and (C) 3′ probes, which identify a 27.7-kb wild-type *CrebH* (wt) fragment and 9.7-kb and 11.3-kb *CrebHloxPneo* (loxPneo) fragments, respectively. (D,E) PCR analyses of ear-punch genomic DNA from *CrebH*$^{+/+}$, *CrebH*$^{+/-}$, *CrebH*$^{-/-}$, *CrebH*$^{+/loxP}$, and *CrebH*$^{loxP/loxP}$ mice using primers shown in panel A. The sizes of *CrebH*$^{+}$ (wt), *CrebH*$^{-}$ (null), and *CrebH*^{loxP} (loxP) amplicons are indicated in base pairs. (F) RT-PCR analyses of RNA extracted from the individual livers of *CrebH*$^{+/+}$(+/+), *CrebH*$^{+/-}$ (+/−), and *CrebH*$^{-/-}$ (−/−) mice using primers that identify *CrebH*, *Pol2* (input RNA control), and *Hnf4α* (hepatocyte control) mRNA. The omission of reverse transcriptase (−RT) and a template (0 DNA) ensured the absence of contaminating DNA.

quences from the Pol2 gene that do not contain HNF4α binding sites, a known HNF4α binding site within the *Apoc3* promoter (H4.21) as well as the HNF4α binding site within the *CrebH* gene could be precipitated from chromatin isolated from fetal livers with an antibody that specifically recognizes HNF4α. Importantly, the levels of products identified when precipitations were performed on brain extracts, which lack HNF4α, or when liver extracts were precipitated with an unrelated antibody (anti-Pes) were low to undetectable. Cumulatively, these data demonstrate that the *CrebH* gene contains an HNF4α recognition element that is occupied by HNF4α in fetal livers.

HNF4α Is Essential for Expression of* CrebH *in the Fetal Liver but Dispensable for Expression in the Small Intestine. To definitively determine whether *CrebH* expression is dependent on HNF4α, we generated E18.5 embryos in which HNF4α was specifically deleted in differentiating hepatocytes (*Hnf4α*$^{loxP/loxP}$*Alfp.cre*),[5,16] colonic epithelial cells (*Hnf4α*$^{loxP/loxP}$*FoxA3.cre*),[17] or small intestine epithelial cells (*Hnf4α*$^{loxP/loxP}$*Villin.cre*), and we measured *CrebH* levels by RT-PCR. Figure 4 shows that although *CrebH* expression was detected in two different control livers, it was not identified in HNF4α-null livers under identical conditions. As expected, *CrebH* was also undetectable in colons regardless of the presence or absence of HNF4α. Somewhat surprisingly, and in contrast to the liver, *CrebH* mRNA was detected in both control and HNF4α-null small intestines. We therefore conclude that HNF4α is dispensable for *CrebH* expression in the gastrointestinal tract but is essential for *CrebH* expression in the liver.

CrebH *Is Dispensable for Hepatogenesis and Hepatocyte Differentiation.* The finding that fetal liver expression of CrebH is dependent on the presence of HNF4α raised the question of whether the absence of CrebH could account for any aspect of the phenotype associated with *Hnf4α*$^{-/-}$ fetal livers. To test this, we

generated two strains of mice, one of which harbored a null allele of *CrebH* (*CrebH*$^{-/-}$) and the other of which harbored a *CrebH* allele that could be conditionally disrupted by expression of Cre recombinase (*CrebH*$^{loxP/loxP}$). Details of the targeting strategy are shown in Fig. 5A. Briefly, embryonic stem cells (*CrebH*$^{+/loxPNeo}$) were generated that contained a single *lox*P site between exons 11 and 12 of *CrebH* and a cassette, which was flanked by *frt* sites, containing a *lox*P site lying 5′ to the neomycin phosphotranferase gene between exons 3 and 4 of *CrebH*. Correct targeting of the *CrebH* locus was confirmed by Southern blot analyses of genomic DNA (Fig. 5B,C), and the altered *CrebH* allele was successfully transmitted through the germ line to generate *CrebH*$^{+/loxPNeo}$ mice. We finally generated mice harboring a null allele of *CrebH* (*CrebH*$^{+/-}$) by mating *CrebH*$^{+/loxPNeo}$ mice with an *EIIa-Cre* transgenic mouse [B6.FVB-Tg(EIIa-cre)C5379Lmgd/J], and this resulted in Cre-mediated recombination between *lox*P sites in the germ line[18] (Fig. 5A,D). We also produced mice containing a conditionally null *CrebH* allele (*CrebH*$^{+/loxP}$) by mating *CrebH*$^{+/loxPNeo}$ mice with mice expressing Flp recombinase from the human *β-actin* gene promoter [B6; SJL-Tg(ACTFLPe)9205Dym/J][19] (Fig. 5A,E).

If CrebH were essential for a central aspect of hepatogenesis, we predicted that *CrebH*$^{-/-}$ embryos would die during late gestation stages. However, crosses of *CrebH*$^{+/-}$ mice yielded *CrebH*$^{-/-}$ offspring at the expected Mendelian ratio as determined with polymerase chain reaction (PCR) analyses of genomic DNA isolated from 162 weanlings. RT-PCR analyses identified *CrebH* mRNA in control livers but not in *CrebH*$^{-/-}$ livers (Fig. 5F), and this is consistent with a loss of *CrebH* activity in the mutant mice. *CrebH*$^{-/-}$ mice were found to be long-lived, fecund, and apparently healthy. Examination of E18.5 embryos revealed no obvious difference between control and mutant embryos (Fig. 6A), and the overall anatomy of the liver and gastrointestinal tract of *CrebH*$^{-/-}$ embryos appeared to be normal. Hematoxylin-eosin (H&E) histological staining and HNF4α immunohistochemistry performed on sections through E18.5 *CrebH*$^{-/-}$ livers found them to be indistinguishable from sections through control livers (Fig. 6B). Finally, oligonucleotide array analyses revealed that mRNA levels were comparable between control and mutant embryos (not shown). Cumulatively, these data demonstrate that *CrebH* is dispensable for hepatogenesis and hepatocyte differentiation in the mouse.

Loss of* CrebH *Results in Reduced Expression of Acute Phase Response Proteins Induced by Tunicamycin. CrebH is a member of the CREB/ATF family of bZip transcription factors that contains a transmembrane domain that directs its localization to the ER.[11,20] Recent

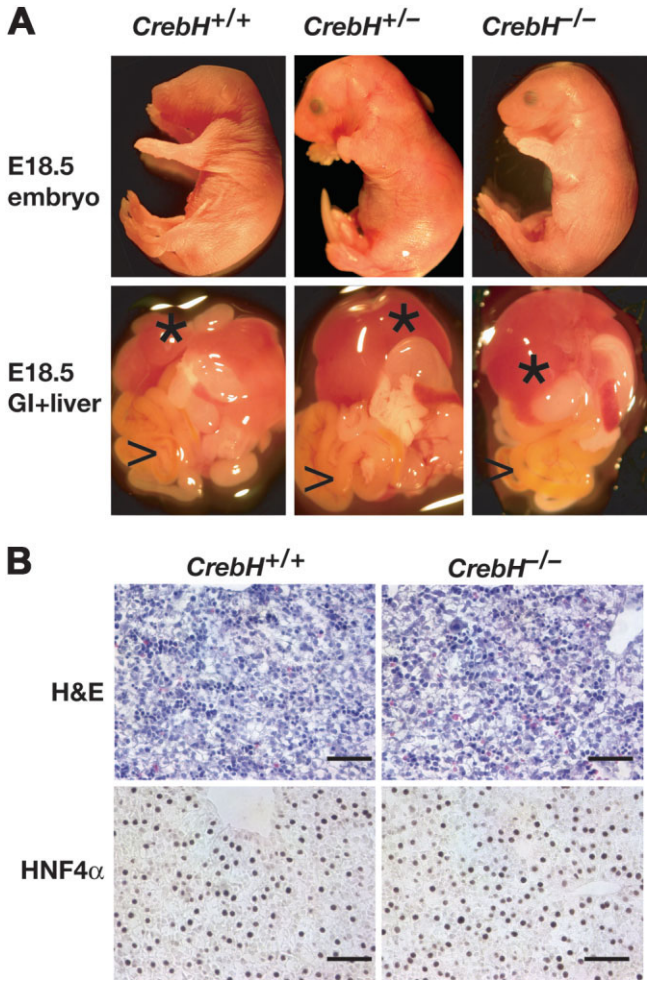


Fig. 6. CrebH is not essential for development of the liver. (A) Micrographs of viscera (lower panels) showing livers (*) and gastrointestinal tracts (>) dissected from E18.5 *CrebH*$^{+/+}$, *CrebH*$^{+/-}$, and *CrebH*$^{-/-}$ embryos (upper panels). (B) Micrographs of sections through *CrebH*$^{+/+}$ and *CrebH*$^{-/-}$ E18.5 livers stained with H&E or for the presence of HNF4α with immunohistochemistry. Scale bar = 100μM.

work has shown that ER stress results in proteolytic cleavage of CrebH, allowing the N-terminus to translocate to the nucleus, where it can activate transcription of target genes including those involved in the acute phase response.[20,21] We therefore examined whether the expression of acute phase genes in response to treatment with tunicamycin, which induces ER stress by blocking glycosylation and protein folding, was affected in *CrebH*$^{-/-}$ mice. Figure 7 shows that the expression of mRNAs encoding the acute phase response proteins C-reactive protein (CRP), serum amyloid P-component (SAP), and serum amyloid A3 (SAA3) was robustly induced in CrebH$^{+/+}$ mice in response to treatment with tunicamycin as expected. However, in contrast to control animals, when CrebH$^{-/-}$ mice were treated with tunicamycin, the expression of each of these acute phase mRNAs was severely reduced. Similar results were obtained for both

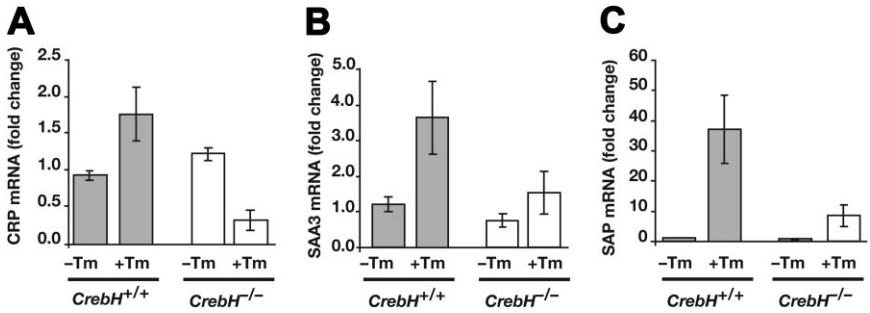


Fig. 7. The response to tunicamycin-induced ER stress is reduced by the loss of CrebH. *CrebH*$^{+/+}$(gray bars) and *CrebH*$^{-/-}$ (white bars) mice were given tunicamycin (2 μg/g of body weight) by intraperitoneal injection. Livers were isolated at 24 hours and processed for quantitative RT-PCR of mRNAs encoding the acute phase proteins (A) CRP, (B) SAA3, and (C) SAP.

liver and serum SAP protein levels (see Supplementary Fig. 8). These results confirm that CrebH is an important component of the systemic inflammatory response to ER stress. Moreover, they imply an indirect role for HNF4α in this response by mediating CrebH expression.

Discussion

In the current analyses, we have identified CrebH as a direct target of HNF4α. Although our data support the view that CrebH expression is strictly dependent on HNF4α in the liver, we have also found that CrebH continues to be robustly expressed in HNF4α-null small intestines. Moreover, although HNF4α is dispensable for intestinal CrebH expression, ChIP analyses have revealed that, like the liver, HNF4α occupies its binding site within the *CrebH* promoter in intestinal tissue (data not shown). These data suggest that the regulation of CrebH by HNF4α is tissue-dependent and importantly that occupancy of a promoter by HNF4α *in vivo* does not necessarily correlate with transcriptional regulation.[1] It is intriguing that CrebH expression is independent of HNF4α in the small intestine yet is strictly dependent on HNF4α in the liver. One possible explanation could be that an intestinal regulatory element exists within the *CrebH* promoter that is controlled specifically by intestinal transcription factors, thereby obviating any requirement for HNF4α. Alternatively, HNF4γ, which in contrast to the liver is robustly expressed in the gut, could potentially compensate for loss of HNF4α in *Hnf4a*$^{lox\mathrm{P}/lox\mathrm{P}}$*Villin.cre* mice. Efforts to address possible redundancy between these two HNF4 family members through the generation of *Hnf4g*$^{-/-}$ animals are currently underway.

To examine the role of CrebH in liver function, we generated *CrebH*$^{-/-}$ mice that were viable and healthy. Although at the onset of the project very little was known about this member of the CREB/ATF family of transcription factors, a number of reports have recently emerged that raise a potential role for this factor in controlling hepatic function.[11,20-22] Most relevant is the finding by Zhang et al.[20] that proinflammatory cytokines or ER stress could activate CrebH through proteolytic cleavage and that depletion of CrebH with short hairpin RNA inhibited expression of acute phase proteins.[20] In addition, CrebH was shown to transactivate expression of a luciferase reporter gene through the *CRP* and *SAP* promoters, and this suggested that CrebH directly regulates expression of these acute phase genes. We therefore examined the expression of mRNAs encoding acute phase response proteins in *CrebH*$^{-/-}$ mice following a treatment with tunicamycin that induced ER stress. We found that expression of CRP, SAP, and SAA3 mRNAs following treatment was severely diminished in *CrebH*$^{-/-}$ animals compared to controls. Although our data confirm an important role for CrebH in the liver, the contribution of CrebH to gut function, particularly in response to ER-induced stress, remains to be determined. However, with the availability of *CrebH*$^{lox\mathrm{P}/lox\mathrm{P}}$ mice, it should be possible to address this issue in future studies.

In conclusion, these results confirm that CrebH has an important role in a pathway that controls expression of acute phase proteins in response to ER stress. Moreover, because hepatic expression of CrebH is dependent on HNF4α, these results demonstrate that HNF4α makes an indirect yet crucial contribution toward the induction of a systemic inflammatory response by ER stress.

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