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Nrf1 and Nrf2 Play Distinct Roles in Activation of Antioxidant Response Element-dependent Genes*[S]

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Nrf1 is a member of the vertebrate Cap'n'Collar (CNC) transcription factor family that commonly contains a unique basic-leucine zipper domain. Among CNC family members, Nrf2 is known to regulate a battery of antioxidant and xenobiotic-metabolizing enzyme genes through the antioxidant response element (ARE). Although Nrf1 has also been shown to bind the ARE, it is unclear whether it plays a distinct role from Nrf2 in regulating genes with this element. To address this issue *in vivo*, we generated mice bearing a hepatocyte-specific disruption of the *Nrf1* gene. Although*Nrf2* knock-out mice did not exhibit liver damage when they were maintained in an unstressed condition, hepatocyte-specific deletion of Nrf1 caused liver damage resembling the human disease non-alcoholic steatohepatitis. Gene expression analysis revealed that the disruption of Nrf1 causes stress that activates a number of ARE-driven genes in an Nrf2-dependent manner, indicating that Nrf2 cannot compensate completely for loss of Nrf1 function in the liver. In contrast, expression of metallothionein-1 and -2 (*MT1* and *MT2*) genes, each of which harbors at least one ARE in its regulatory region, was decreased in the *Nrf1*-null mutant mice. Whereas Nrf1 and Nrf2 bound the *MT1* ARE with comparable affinity, Nrf1 preferentially activated the reporter gene expression through the *MT1* ARE. This study has, thus, identified the first ARE-dependent gene that relies exclusively on Nrf1, suggesting that it plays a distinct functional role in regulating ARE-driven genes.

One of the unique features of transcription factors is that they can be categorized into structurally related groups (or families) based on their DNA binding motifs. It appears that in addition to their common basic functions each member within a specific transcription factor family is likely to have acquired novel properties during its molecular evolution. Consequently, it is probable that a family of transcription factors collectively possesses abilities that enable individual members to fine-tune the expression of target genes during disparate biological processes through a common *cis*-acting element.

The Cap'n'Collar (CNC)[3] family of transcription factors has been well described. It comprises four closely related factors, Nrf1, Nrf2, Nrf3, and p45 NF-E2 (1, 2). These CNC members contain a CNC domain juxtaposed with a conserved basic region-leucine zipper (bZip) domain. The CNC domain is represented in an evolutionally distant homologue, SKN1 in the nematode *Caenorhabditis elegans* (3). Although SKN1 binds to DNA as a monomer, all vertebrate CNC members each heterodimerize with one of three small Maf proteins, and the resulting heterodimers bind to the Maf recognition element (TGCTGA(G/C)TCAGCA) or related sequences. Because the vertebrate CNC members cannot bind to Maf recognition element sequences as monomers, small Maf proteins are indispensable partners of CNC bZip transcription factors (1, 2).

The Maf recognition element-related sequence that is referred to as an antioxidant or electrophile response element (ARE/EpRE; hereafter designated ARE for simplicity) has been identified in the transcriptional regulatory regions of antioxidant and xenobiotic-metabolizing enzyme genes (1, 2) and is thought to account for the coordinated up-regulation of these genes during oxidative stress. The ARE has been shown to recruit Nrf2 and small Maf proteins (1, 2). In *Nrf2*-null mutant mice the up-regulation of many antioxidant and detoxification enzyme genes has been found to be severely impaired (4). Nrf1 has also been shown to contribute to the regulation of ARE-dependent genes (5, 6). Unlike *Nrf2*-null mice, *Nrf1*-null mice die by embryonic day 13.5 (E13.5) due to anemia (7). Furthermore, *Nrf1*:*Nrf2* compound mutant mice die by E11.5, suggesting that Nrf2 can compensate, albeit partially, for the absence of Nrf1 in embryonic mice (8). Based on the observation that an evolutionally distant CNC homolog SKN1 also contributes to the induction of antioxidant genes (9), it can be hypothesized that

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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[3] The abbreviations used are: CNC, Cap'n'Collar; NASH, non-alcoholic steatohepatitis; ARE, antioxidant response element; ER, endoplasmic reticulum; MT, metallothionein; RT, reverse transcription; NQO1, NAD(P)H dehydrogenase quinone 1; GCLC, glutamate-cysteine ligase catalytic subunit; HO-1, heme oxygenase-1; GSTP1, glutathione *S*-transferase π1.

the regulation of antioxidant genes is one of the original functions descended from a common ancestral CNC protein.

It is clear that Nrf1 and Nrf2 have acquired specific functions during their molecular evolution. It has been reported that hepatocyte-specific conditional targeting of the *Nrf1* gene (Nrf1 cKO) causes a liver pathology resembling human non-alcoholic steatohepatitis (NASH) (10); this previous study also reported that the expression of several ARE-dependent genes was decreased in the livers of Nrf1 cKO mice, suggesting that Nrf1 has an overlapping function with Nrf2 in the regulation of antioxidant and detoxifying genes. In stark contrast, however, *Nrf2*-null mutant mice do not show any liver damage when housed in an unstressed environment (11). Thus, perturbations in the expression of antioxidant genes in Nrf1 cKO mice appear unlikely to account for the NASH-like disorder. Collectively, these observations suggest that Nrf1 possesses unique functions that are not exhibited by Nrf2. To execute its unique activity, Nrf1 protein possesses several distinct domains that are conserved among cross-species Nrf1 homologues (12). For instance, Nrf1 has an endoplasmic reticulum (ER) targeting sequence in its N-terminal domain that is responsible for it being anchored to the ER membrane and an Asn/Ser/Thr-rich (NST) domain through which it is glycosylated within the ER (13). It has also been reported that Nrf1 is cleaved and translocated from the ER to the nucleus in response to ER stress (14), although the functional contribution of Nrf1 to the ER stress response has not been well described.

Because the distinct phenotypes of Nrf1 and Nrf2 knock-out mice are highly likely to be caused by differences in the expression of their relevant target genes, it is necessary to examine gene expression profiles in mutant mice to understand the molecular basis for the severe consequences after disruption of Nrf1. In this study we have, therefore, independently generated hepatocyte-specific *Nrf1* cKO mice and carried out comprehensive gene expression analyses. To our surprise, expression of canonical Nrf2 target genes in the liver was induced by the loss of Nrf1 in Nrf2-dependent manner, indicating that dysregulation of Nrf1 target genes causes an activation of the Nrf2-stress response pathway. We also found that several ARE-driven genes were down-regulated in the Nrf1 cKO mice compared with control mice. Among them, we found that expression of metallothionein-1 (*MT1*) and -2 (*MT2*) genes is intimately dependent on Nrf1 but not on Nrf2. A reporter co-transfection assay showed that Nrf1 preferentially activates the *MT1* ARE. Moreover, in livers from *Keap1* knockdown mutant mice ($Keap1^{kd/kd}$), where Nrf2 activates its target genes without any stimuli (15), no significant induction of *MT1* expression was observed. These results, thus, demonstrate that Nrf1 has acquired specific roles distinct from those of the other CNC family members.

EXPERIMENTAL PROCEDURES

Hepatocyte-specific Disruption of the Nrf1 Gene—129Sv mouse *Nrf1* genomic DNA was isolated from a BAC clone containing the murine *Nrf1* gene from the BACPAC Resources Center at Children's Hospital Oakland Research Institute (Oakland, CA). We constructed a triple-loxP *Nrf1* targeting vector to generate a floxed mouse *Nrf1*-targeted locus consisting of a loxP site inserted into the third *Nrf1* intron and a floxed Neomycin cassette downstream of exon IV (Fig. 1*A*). A splicing acceptor-fused internal ribosomal entry site-enhanced green fluorescence protein (Invitrogen) cassette was also incorporated into the construct at a site 3′ of the Neomycin cassette. A diphtheria toxin A cassette was placed outside of the *Nrf1* gene homology region to allow negative selection. From PCR screening, we identified one clone that carried a homozygously recombined allele.

The existence of ES cells with the floxed *Nrf1*-targeted locus was confirmed by Southern blot analysis of NsiI- and SpeI-digested genomic DNA using a 5′ probe located outside the targeting vector. The clone showed positive 19.4- and 13.1-kilobase fragments. The targeted ES cells were microinjected into blastocysts, and chimeric mice were crossed with wild-type animals to generate $Nrf1^{flox/+}$ mice. Hepatocyte-specific deletion of the floxed *Nrf1* allele was accomplished through breeding with the albumin-Cre transgenic mice (*Alb-Cre*, purchased from The Jackson Laboratory (Bar Harbor, ME)). The *Alb-Cre* mice were crossed with $Nrf1^{flox/+}$ mice to generate $Nrf1^{flox/+}$:*Alb-Cre* mice, which were then crossed with each other to generate hepatocyte-specific *Nrf1*-deficient ($Nrf1^{flox/flox}$:*Alb-Cre*) mice.

Generation of Compound Mutant Mice—After mating $Nrf1^{flox/flox}$:*Alb-Cre* mice with either *Nrf2* homozygous ($Nrf2^{-/-}$) mutant mice (4) or *Keap1* knockdown mutant mice (15), $Nrf1^{flox/+}$:$Nrf2^{+/-}$:*Alb-Cre* mice or $Nrf1^{flox/+}$:$Keap1^{kd/+}$ mice were obtained. These offspring were intercrossed to generate the following compound mutant mice: $Nrf1^{flox/flox}$:$Nrf2^{-/-}$:*Alb-Cre* and $Nrf1^{flox/flox}$:$Keap1^{kd/kd}$.

RNA Extraction, RT-PCR, and Quantitative RT-PCR—Total RNA was extracted from livers of three 8-week-old $Nrf1^{flox/flox}$:*Alb-Cre* mice and $Nrf1^{flox/flox}$ mice using ISOGEN (Nippon Gene), and cDNA was synthesized using random hexamer oligonucleotide primers in reactions that were performed according to protocols recommended by the manufacturer. PCR primers for Nrf1 were as follows: Nrf1-F (5′-GGT GCC TAG TGA GAG TGA GTC CCC C-3′) and Nrf1-R (5′-TCG GGG CTG AAG AGG GAG AAG TC-3′). Primers specific to hypoxanthine phosphoribosyltransferase (*HPRT*) have been described earlier (16). Quantitative RT-PCR was performed using an ABI Prism 7300 or 7700 (Applied Biosystems) instrument as previously reported (17). The primer and probe sequences used to detect NAD(P)H dehydrogenase quinone 1 (*NQO1*), the glutamate-cysteine ligase catalytic subunit (*GCLC*), heme oxygenase-1 (*HO-1*), and glutathione *S*-transferase π1 (*GSTP1*) mRNA have been described previously (18, 29). *Nrf1*, *MT1*, and *MT2* mRNAs were quantified using the following primer and probe sequences: *Nrf1*-sense (5′-TTG GAA CAG CAG TGG CAA GA-3′), *Nrf1*-antisense (5′-CTC ACT TGC TGA TGT ATT TAC TTC CAT-3′), *Nrf1*-probe (5′-FAM CTC ATG TCC ATC ATG GAA ATG CAG GC TAMRA-3′), *MT1* sense (5′-AAC TGC TCC TGC TCC AC-3′), *MT1* antisense (5′-GCC CTG GGC ACA TTT GG-3′), *MT1* probe (5′-FAM CTC CTG CAA GAA GAG CTG CTG CTC CT TAMRA-3′), *MT2* sense (5′-TCC TGT GCC TCC GAT GGA TC-3′), *MT2* antisense (5′-GTC GGA AGC CTC TTT GCA GA-3′), *MT2* probe (5′-FAM AAA GCT GCT GCT

CCT GCT GCC CC TAMRA-3′). The rRNA primers and probe were purchased from Applied Biosystems.

Microarray Analysis—Total RNA samples were purified using ISOGEN before being processed and hybridized to the Affymetrix Mouse Genome 430 2.0 Array (Affymetrix). Experimental procedures for the Gene Chip analyses were performed according to the Affymetrix Technical Manual.

Histochemical Analysis—Livers were fixed with Mildform (Wako) and embedded in paraffin using standard procedures. Sections (4 μm) were cut and stained with hematoxylin and eosin. To visualize hepatic lipid content, livers were fixed with 4% paraformaldehyde and embedded in OCT (Tissue Tek). The frozen sections (5 μm) were stained with Oil Red O (Muto Pure Chemicals) and counterstained with hematoxylin.

Clinical Biochemistry—Plasma was obtained by centrifugation at 4 °C and either analyzed immediately or stored at −20 °C. Plasma alanine aminotransferase and aspartate transaminase activities were measured with an automated biochemical analyzer, DRI-CHEM 7000V (Fuji Film, Japan).

Electrophoretic Mobility Shift Assay—Nrf1-CT, Nrf2-CT (19), and MafG (1–123) (20) were tagged with six histidine residues at their N termini and purified by using nickel-chelating affinity chromatography. The oligonucleotide *MT1*-ARE-F (5′-CGC GCG GCT CTG CCA AGG ACG CGG GGC GCG TGA CTA TGC GTG GGC T-3′) was radiolabeled with ^{32}P and annealed with the complementary strand oligonucleotide *MT1*-ARE-R (5′-CTA GAG C CC ACG CAT AGT CAC GCG CCC CGC G TC CTT GGC AGA GCC G-3′). To generate unlabeled competitors, a pair of oligonucleotides, *MT1*-ARE-F and *MT1*-ARE-R, (above; wild type competitor) and their corresponding mutant oligonucleotides, *MT1*-mARE-F (5′-CGC GCG GCT CTG CCA AGG ACG CGG GGC GCG TGA CTA TAA GTG GGC T-3′) and *MT1*-mARE-R (5′-CTA GAG CCC TTG CAT AGT CAC GCG CCC CGC GTC CTT GGC AGA GCC G-3′) were annealed. Where required, a 100-fold excess of competitor oligonucleotide was added to individual reaction mixtures. Incubation of the probe and recombinant proteins with or without unlabeled competitor was carried out as described previously (21). Thereafter, the protein-DNA complexes and free probe were resolved by electrophoresis on a 5% polyacrylamide (29:1) gel in 1× Tris borate EDTA buffer.

Co-transfection Transactivation Assay—The mouse *MT1* gene promoter along with its ARE-containing 153-bp upstream region (a kind gift from Dr. G. K. Andrews, University of Kansas Medical Center, Kansas City) (22) was inserted into the pGL2-basic vector (pMT1 ARE-Luc). A reporter construct lacking the putative ARE was generated by deleting sequences CGTGAC-TATGCG (−99 to −88, relative to the transcriptional start site) from the pMT1 ARE-Luc reporter plasmid. A cDNA encoding mouse Nrf1 lacking amino acids 1–30 was inserted into pcDNA3.1 (Promega), generating pcDNA3.1FLAG-Nrf1Δ30 (*i.e.* Nrf1 lacking its ER localization signal (14)). Hepa1c1c7 (Hepa1) cells were co-transfected with pMT1 ARE-Luc or pNQO1 ARE-Luc together with or without 100 or 200 ng of each pcDNA3.1FLAG-Nrf1Δ30, and pcDNA3.1FLAG-Nrf2, respectively, using the Lipofectamine Plus (Invitrogen) according to the manufacture's protocol. All transfection mixtures included 0.001 μg of the Renilla luciferase reporter, pRL-EF. Transfected cells were incubated for 24 h before the expression of both firefly and Renilla luciferase was quantified using a dual luciferase reporter assay (Promega); firefly luciferase activity was normalized to co-transfected Renilla luciferase activity for transfection efficiency.

Western Blot Analysis—The preparation of nuclear lysates (23) from Hepa1 cells that had been transfected with pcDNA3.1FLAG-Nrf1Δ30 or pcDNA3.1FLAG-Nrf2 were subjected to immunoblot analysis as described previously. Membranes were probed with antibodies directed against FLAG-M2 (Sigma-Aldrich). To ensure equal loading, membranes were stripped and re-probed with anti-lamin antibodies (Santa Cruz). A horseradish peroxidase-conjugated secondary antibody (Zymed Laboratories Inc.) was used. Blots were developed by enhanced chemiluminescence.

RESULTS

Generation of Conditional Nrf1 Knock-out Mice—To help further define the *in vivo* functions of Nrf1, we generated conditional *Nrf1* mutant mice using the Cre-loxP system. A targeting vector was constructed in which the coding region of exon IV was flanked with loxP sequences (Fig. 1*A*). Because this exon encodes the bZip DNA binding domain, we expected that the Cre-loxP recombination would generate a truncated form of Nrf1 that was incapable of binding DNA and transactivating gene expression. We also inserted an internal ribosomal entry site-enhanced green fluorescence protein cassette in the 3′ region of *Nrf1* so that its expression could be evaluated by monitoring green fluorescent protein.

We confirmed homologous recombination between the targeting vector and the *Nrf1* locus in mouse ES cells by Southern blotting (Fig. 1*B*). NsiI-digested genomic DNA was hybridized to a probe complementary to the 5′ region of the *Nrf1* gene. A homologous recombinant clone was identified by the presence of an additional short DNA fragment created by the presence of a SpeI site in the flanking region of the loxP element.

Chimeric mice were obtained by microinjecting *Nrf1*$^{flox/+}$ ES cells into mouse blastocysts, and the resulting animals were crossed with wild-type C57BL/6J mice to achieve germ line transmission of the floxed *Nrf1* allele. In preliminary experiments *Nrf1*$^{flox/flox}$ mice were crossed with *AYU1-Cre* mice, which expressed Cre recombinase ubiquitously and displayed embryonic lethality by E13.5 (data not shown), consistent with a previous *Nrf1* gene knock-out analysis (7). We also confirmed that insertion of loxP sites into the wild-type allele did not alter significantly the expression of *Nrf1* in livers of adult mice (Fig. 1*C*).

Hepatocyte-specific Nrf1 Knock-out Mice Show Liver Damage—To generate a hepatocyte-specific deletion of Nrf1 in mice, we crossed *Nrf1*$^{flox/flox}$ mice with Alb-Cre transgenic mice, yielding *Nrf1*$^{flox/flox}$:Alb-Cre mice. PCR genotyping results of the Nrf1-floxed allele and Nrf1-deleted allele with mouse liver genomic DNA are shown in Fig. 2*A*. The Alb-Cre-mediated excision of the floxed allele appeared to be essentially complete, and RT-PCR analysis revealed that Nrf1 mRNA levels were severely compromised in livers of *Nrf1*$^{flox/flox}$:Alb-Cre mutant mice (Fig. 2*B*).

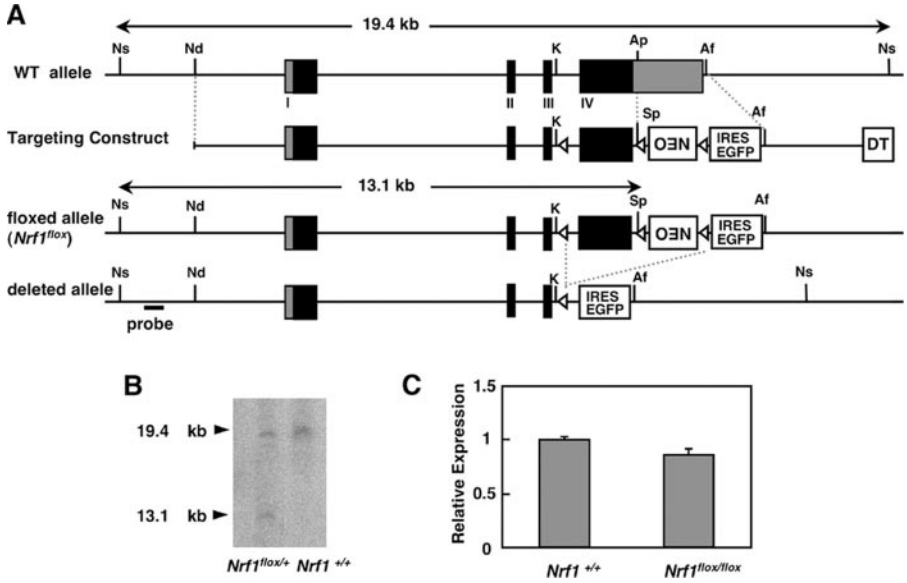

FIGURE 1. **Generation of *Nrf1*-floxed mice.** *A*, the targeting strategy is illustrated. Structures of *Nrf1* allele (wild type (*WT*) allele), *Nrf1* gene-targeting construct, *Nrf1*-floxed allele, and *Nrf1*-deleted allele after Cre-mediated recombination are shown. Coding exons of the mouse *Nrf1* gene are shown with *filled boxes*, untranslated regions are shown with a *gray box*, and loxP sites are indicated with *open arrowheads*. *Ns*, *Nd*, *K*, *Ap*, *Sp*, and *Af* stand for NsiI, NdeI, KpnI, ApaLI, SpeI, and AflII sites, respectively. *kb*, kilobase. *B*, Southern blot analysis of the floxed Nrf1 allele. NsiI-NsiI fragment specific to wild-type allele (19.4 kilobases) and NsiI-SpeI fragment specific to floxed allele (13.1 kilobases) are shown as *double-headed arrows* in *A*. The probe used is shown as a *bar* in *A*. *C*, *Nrf1* mRNA expression level in the liver. Quantitative RT-PCR was performed to examine the expression of *Nrf1* mRNA in wild type and *Nrf1*$^{flox/flox}$ mice. The expression of *Nrf1* mRNA in wild-type mouse was set as 1. The *error bars* represent S.D.

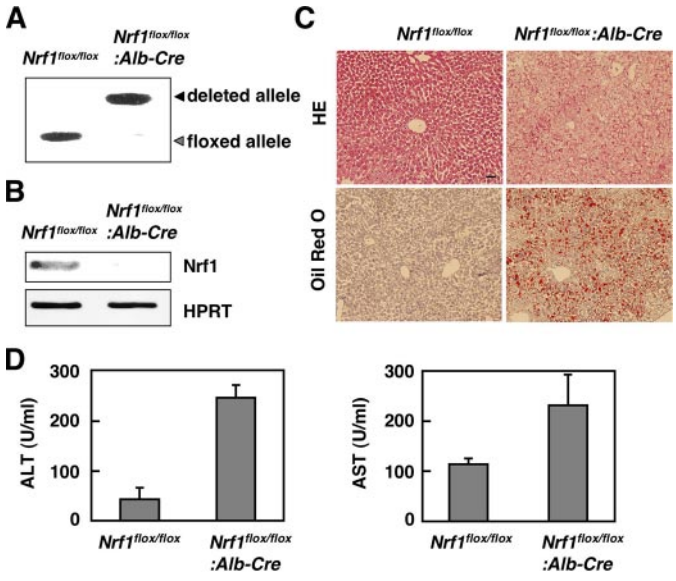

FIGURE 2. **Cre-mediated hepatocyte-specific disruption of *Nrf1* gene.** *A*, PCR detection of the *Nrf1*-floxed and -deleted alleles using mouse liver genomic DNA. *B*, *Nrf1* mRNA was detected by RT-PCR in the liver of *Nrf1*$^{flox/flox}$ and *Nrf1*$^{flox/flox}$*:Alb-Cre*. Hypoxanthine phosphoribosyltransferase (*HPRT*) expression level was examined as a loading control. *C*, liver sections stained with hematoxylin and eosin (*HE*) in the *upper panels*. The *scale bar* corresponds to 300 μm. To detect the accumulated lipid, the liver sections were stained with Oil Red O, as shown in the *lower panels*. *D*, the serum alanine aminotransferase (*ALT*) and aspartate transaminase activities of *Nrf1*$^{flox/flox}$ and *Nrf1*$^{flox/flox}$*:Alb-Cre* mice at 8 weeks of age were examined. The *error bars* represent S.D. *U*, units.

Histological examination of liver sections from 8-week-old *Nrf1*$^{flox/flox}$:Alb-Cre mutant mice stained with hematoxylin and eosin revealed that they contained many lipid vacuoles (Fig. 2*C*, *upper panels*). To confirm the lipid accumulation, we also stained frozen sections with Oil Red O (Fig. 2*C*, *lower panels*). The results clearly demonstrated that the hepatocytes of *Nrf1*$^{flox/flox}$:Alb-Cre mice accumulated a massive amount of lipid when compared with control mice. In clinical biochemistry analyses of mice at 8 weeks of age, plasma alanine aminotransferase and aspartate transaminase levels in *Nrf1*$^{flox/flox}$:Alb-Cre mice were significantly higher than those in control mice (Fig. 2*D*). These results support the previous report that liver-specific knock-out of Nrf1 gene causes pathologic changes in the liver that resemble human NASH (10).

Inducible Expression of Cytoprotective Genes in Nrf1$^{flox/flox}$*:Alb-Cre Mouse Liver in a Nrf2-dependent Manner*—We performed microarray analyses of RNA from livers of *Nrf1*$^{flox/flox}$*:Alb-Cre* mice using livers from *Nrf1*$^{flox/flox}$ animals as controls. Because it has been reported that Nrf1 contributes to the expression of cytoprotective enzyme genes that are known to be regulated by Nrf2, we examined whether the expression of Nrf2 target genes was affected in *Nrf1*$^{flox/flox}$*:Alb-Cre* mouse livers. The results revealed that the expression of a set of Nrf2/ARE-regulated genes is increased rather than decreased even in the absence of Nrf1 (Table 1).

To verify these microarray data, we selected four ARE-dependent genes (*NQO1*, *GSTP1*, *GCLC*, and *HO-1*) and performed quantitative RT-PCR analysis. We found that the basal expression level of these genes was increased in *Nrf1*$^{flox/flox}$*:Alb-Cre* mouse livers when compared with control (*Nrf1*$^{flox/flox}$) mouse livers (Fig. 3). Thus, *Nrf1*-deficiency resulted in up-regulation of at least some Nrf2-target genes.

To examine whether the increased expression of these cytoprotective genes depends on Nrf2, we generated a compound mutant mouse line bearing a global deletion of *Nrf2* concomitant with hepatocyte-specific deletion of *Nrf1*. The increase in expression of these four Nrf2 target genes was completely abrogated in the *Nrf1*:*Nrf2* compound knock-out mouse liver. This result suggested that the expression of antioxidant and xenobiotic-metabolizing enzyme genes is up-regulated in an Nrf2-dependent manner in response to endogenous stress caused by the lack of Nrf1. An important observation is that Nrf1 does not contribute to the basal expression of these four genes even in the absence of Nrf2, thereby suggesting that, at least under steady state conditions, Nrf1 does not contribute to the regulation of Nrf2 target genes (Fig. 3).

Identification of Nrf1-dependent Genes in Liver—In the microarray analyses we examined genes that were down-regulated in the *Nrf1*$^{flox/flox}$*:Alb-Cre* mouse livers. We found that 52

TABLE 1

Effect of Nrf1 deficiency on expression levels of typical Nrf2-dependent genes

Each value represents the -fold increase of the gene expression levels in *Nrf1*$^{flox/flox}$:*Alb-Cre* mice relative to those in *Nrf1*$^{flox/flox}$ mice, which was calculated from the raw data of the microarray analysis.

Description of genes	Accession number	-Fold increase
Glutathione *S*-transferase, α1/2	NM_008182	2.90
Glutathione *S*-transferase, μ2	NM_008183	2.46
Glutathione *S*-transferase, ζ1	AB041613	2.27
Glutathione *S*-transferase, α4	NM_010357	2.05
Glutathione *S*-transferase, μ3	J03953	2.01
Glutathione *S*-transferase, μ6	NM_008184	1.95
Glutathione *S*-transferase, μ5	NM_010360	1.88
Glutathione *S*-transferase, κ1	AK002661	1.76
Glutathione peroxidase 2	NM_030677	1.75
Glutathione *S*-transferase, α3	AI172943	1.72
Glutathione *S*-transferase, μ1	NM_010358	1.53
Glutathione *S*-transferase, π1	NM_013541	1.41
Peroxiredoxin 1	AV124700	1.34
NAD(P)H dehydrogenase, quinone 1	AV158882	1.11
Glutathione peroxidase 1	BI219063	1.10
Thioredoxin 1	NM_011660	1.07
Ferritin light chain 1	NM_010240	0.89
Heme oxygenase 1	NM_010442	0.74
Glutamate-cysteine ligase, modifier subunit	NM_008129	0.73
Glutamate-cysteine ligase, catalytic subunit	BC019374	0.65

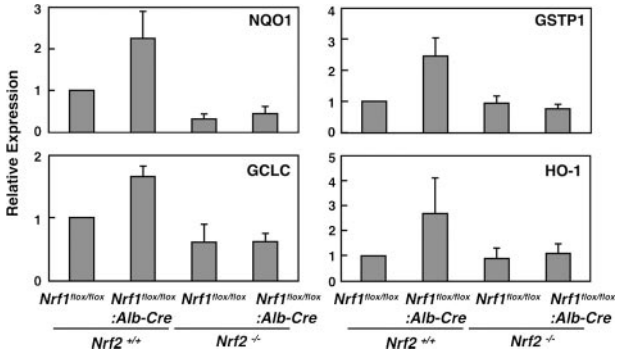

FIGURE 3. **Nrf1 deficiency results in up-regulation of Nrf2-target genes, *NQO1, GSTP1, GCLC,* and *HO-1*.** Expression of xenobiotic metabolizing enzymes and antioxidant enzymes were examined by quantitative RT-PCR. The four genes were selected based on the result of the microarray analysis. RNA samples were prepared from livers of *Nrf1*$^{flox/flox}$, *Nrf1*$^{flox/flox}$:*Alb-Cre*, *Nrf1*$^{flox/flox}$:*Nrf2*$^{-/-}$, and *Nrf1*$^{flox/flox}$:*Nrf2*$^{-/-}$:*Alb-Cre* mice. The expression level of each mRNA was normalized to the rRNA abundance. The expression of each gene in *Nrf1*$^{flox/flox}$ mouse was set to 1. The *error bars* represent S.E. of mean of three independent experiments.

genes were decreased more than 3-fold in the *Nrf1*$^{flox/flox}$:*Alb-Cre* mouse livers when compared with the control mouse livers. We categorized these genes into 11 groups according to their function (Table 2). To our surprise, only three of the 52 genes could be categorized as belonging to the xenobiotic metabolism group (*i.e. MT1*, *MT2*, and sulfotransferase family 3A member 1). On the other hand, we found that many of the hepatic genes that were down-regulated in *Nrf1*-null livers contributed to the maintenance of normal homeostatic processes. This group of genes includes glycosylation-related proteins, a chaperone, and various metabolic enzymes. These results, thus, indicate that Nrf1 is involved in the regulation of a number of important biological functions in the liver, which are distinct from those of Nrf2.

Metallothionein Genes Are Down-regulated in Nrf1$^{flox/flox}$*:Alb-Cre Mice*—Among the Nrf1-dependent genes identified in the microarray study, *MT1* and *MT2* have been reported to contain at least one ARE in their regulatory regions (22). To verify these findings, we performed quantitative RT-PCR analyses and confirmed that the expression of both *MT1* and *MT2*

TABLE 2

Genes whose expression levels were decreased in *Nrf1*$^{flox/flox}$:*Alb*-Cre mice compared with *Nrf1*$^{flox/flox}$ mice

Each value represents the -fold decrease of the gene expression levels in *Nrf1*$^{flox/flox}$:*Alb-Cre* mice relative to those in *Nrf1*$^{flox/flox}$ mice, which was calculated from the raw data of the microarray analysis. The genes are categorized and listed if they were reduced by >3-fold except for those with unknown functions. Genes in bold type means that the gene has potential AREs in their promoter-proximal regions (see supplemental Table I).

Gene category and description	Accession number	-Fold decrease
Xenobiotic metabolism		
Metallothionein 2	AA796766	8.03
Metallothionein 1	BC027262	7.76
Sulfotransferase family 3A, member 1	NM_020565	3.07
Metabolic enzyme		
Pyruvate dehydrogenase kinase, isoenzyme 4	NM_013743	3.68
Acyl-CoA thioesterase 1	NM_012006	3.25
Chaperone		
Heat shock protein 8	BB764222	3.07
Glycosylation		
Glycosylphosphatidylinositol anchor attachment protein 1	NM_010331	3.32
Phosphatidylinositol glycan, class O	BB546713	3.04
Transporter		
ATP-binding cassette, sub-family F (GCN20), member 1	AV309591	11.85
Major facilitator superfamily domain containing 3	BC019171	7.57
Sideroflexin 2	NM_053196	3.13
Cell cycle and differentiation		
Growth arrest and DNA-damage-inducible gene 45γ	AK007410	15.55
Cdc42-binding protein kinase α	BM117074	4.01
Bcl-associated death promoter	NM_007522	3.91
Cyclin-dependent kinase 5	NM_007668	3.02
Signal transduction		
Signal peptide peptidase 3	BC023131	7.28
Serine/threonine kinase 24 (STE20 homolog, yeast)	BG060677	4.71
Rho guanine nucleotide exchange factor (GEF) 12	AI481688	3.17
Calcium/calmodulin-dependent protein kinase kinase 2β	BI157430	3.07
TRAF family member-associated NF-κβ activator (TANK)-binding kinase 1	NM_019786	3.07
SCY1-like 2 (*Saccharomyces cerevisiae*)	BM249802	3.05
Cytoskeletal organization		
Rho GTPase-activating protein 6	AF177664	4.32
Dopachrome tautomerase	NM_010024	3.03
Nuclear proteins		
Cysteine and glycine-rich protein 1	BF124540	3.78
Ribonuclease P 40 subunit (human)	R75260	14.53
GTP-binding protein 2	NM_019581	4.85
Coiled-coil domain containing 91	AK007017	3.85
Small nuclear ribonucleoprotein N	NM_033174	3.68
Ribonuclease H2, subunit B	AU017373	3.54
Histone 2, H3c1 /histone 2, H2aa1	BC010564	3.00
Immunological proteins		
Histocompatibility 2, D region locus 1	L23495	4.93
Interleukin 1 receptor, type I	NM_008362	3.68
Miscellaneous		
C-type lectin domain family 2, member h	AF350410	13.37
Crystallin, μ	NM_016669	9.92
Zinc finger, A20 domain containing 2	AA124553	3.53
Zinc finger protein 259	AK016551	3.47
B-cell CLL/lymphoma 7B	NM_009745	3.36
Praja1, RING-H2 motif-containing	BM199789	3.20
EH-domain containing 1	NM_010119	3.19
Zinc finger, AN1 type domain 2B	BC011495	3.17
Rhomboid domain containing 2	BB233055	3.14
Aminolevulinic acid synthase 1	AI255644	3.11
Myocyte enhancer factor 2D	BG067616	3.09

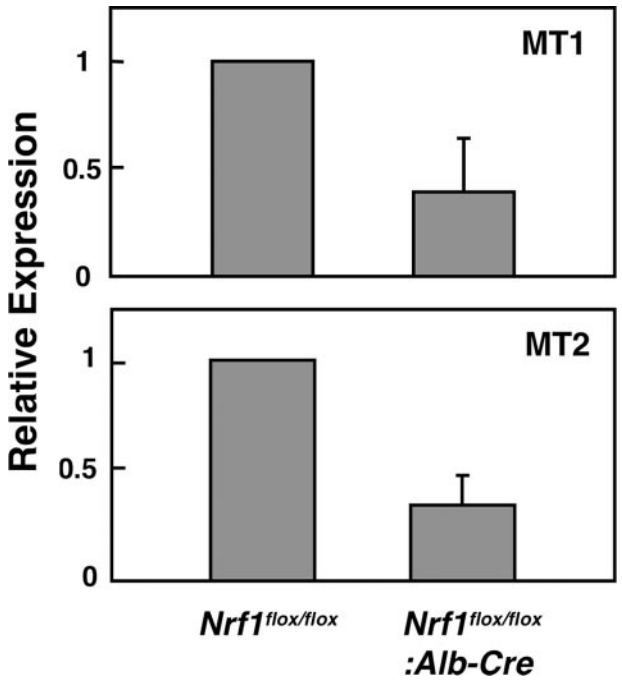

FIGURE 4. **Expression of *MT1* and *MT2* genes depends on Nrf1.** The expression of *MT1* and *MT2* genes was examined by quantitative RT-PCR. RNA samples are prepared from *Nrf1*$^{flox/flox}$ and *Nrf1*$^{flox/flox}$:*Alb-Cre* mice. The expression level of each mRNA was normalized to the rRNA abundance. The expression of each gene in *Nrf1*$^{flox/flox}$ mouse was set to 1. The *error bars* represent S.D.

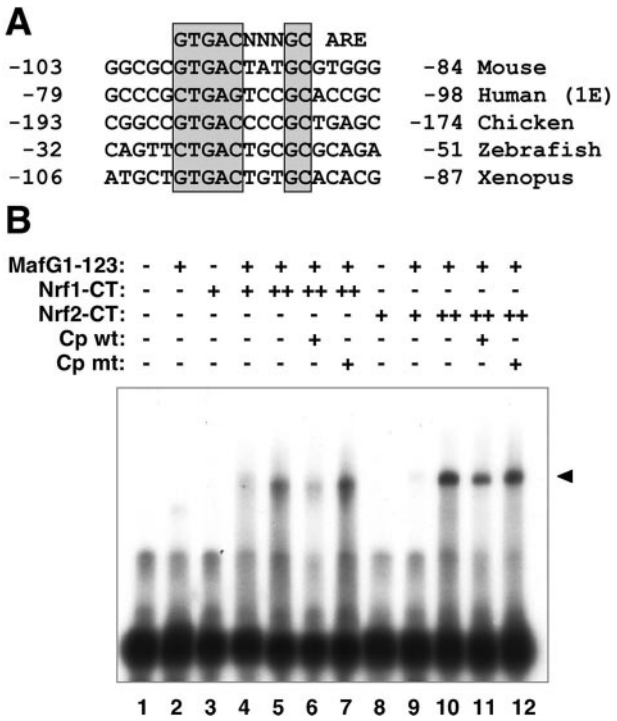

FIGURE 5. **Binding of Nrf1 and Nrf2 to *MT1* ARE.** *A*, an alignment of AREs found in the promoter regions of the mouse, human (1E), chicken, *Xenopus*, and zebrafish *MT1* genes. *Numbers* indicate the position relative to the transcriptional initiation site. ARE consensus sequences are *shaded*. *B*, electrophoretic mobility shift assay was performed with *MT1* ARE probe. DNA binding ability of the heterodimers consisting of Nrf1-CT/MafG1–123 or Nrf2-CT/MafG1–123 was examined. Constant amounts of MafG1–123 and increasing amounts of Nrf1-CT (*lanes 3–7*) and Nrf2-CT (*lanes 8–12*) were incubated with *MT1* ARE probe. The *black arrowhead* indicates the DNA binding activity of heterodimeric proteins. A 100-fold molar excess of unlabeled wild-type *MT1* ARE probe (*Cp wt*) (*lanes 6* and *11*) or mutant *MT1* ARE probe (*Cp mt*) (*lanes 7* and *12*) was added for competition reactions.

genes was compromised in *Nrf1*$^{flox/flox}$:*Alb*-Cre mouse livers when compared with their expression levels in control mice (Fig. 4). It is well known that the expression of MT is induced in response to heavy metals and that this process is mediated by the zinc-finger transcription factor MTF1 through the metal response element in gene promoters (24). Our microarray data showed that MTF1 expression level did not change in the *Nrf1*$^{flox/flox}$:*Alb*-Cre mouse livers (results not shown), indicating that the reduction of *MT1* in the *Nrf1*$^{flox/flox}$:*Alb*-Cre mouse livers was not caused by down-regulation of MTF1. Because the other ARE-dependent genes were induced in *Nrf1*$^{flox/flox}$:*Alb*-Cre mice in an Nrf2-dependent manner (Fig. 3), these results raised the possibility that in mouse liver the *MT1* and *MT2* genes are preferentially and specifically regulated by Nrf1, but not by Nrf2, through the ARE in their regulatory regions.

MT1 ARE Is Preferentially Regulated by Nrf1 Not by Nrf2—Transcriptional control of the *MT1* gene has been extensively studied, especially with regard to transcription factor MTF1 and its binding to the metal response element sequence (24). In addition to this metal response element, it has been reported that the *MT1* gene has a conserved ARE in its regulatory region (25). We also confirmed that AREs are present in the promoter regions of orthologous vertebrate *MT* genes, such as those from mouse, human, chicken, *Xenopus*, and zebrafish (Fig. 5*A*). However, contribution of the CNC family factors to the regulation of *MT1* expression has not been well documented.

To examine whether Nrf1 and Nrf2 bind to the mouse *MT1* ARE, we performed electrophoretic mobility shift assay analyses. For this purpose, truncated Nrf1, Nrf2, and MafG proteins containing their DNA binding domains (*i.e.* Nrf1-CT, Nrf2-CT, and MafG1–123) were prepared in a bacterial expression system. We found that both Nrf1-MafG and Nrf2-MafG heterodimers could bind to the *MT1* ARE with similar affinity (Fig. 5*B*). The observed binding activity was specific because it was competed by 100-fold molar excess of an unlabeled wild-type ARE probe but not by a mutant ARE probe (Fig. 5*B*). We conclude that Nrf1 and Nrf2 exhibit similar binding affinities for the *MT1* ARE.

We then tested the possibility that Nrf1 and Nrf2 differentially activate transcription through the *MT1* ARE. We performed a reporter co-transfection/transactivation assay. The *MT1* gene regulatory region containing the ARE was linked to a luciferase gene, and the reporter construct was transfected into Hepa1 cells simultaneously with either FLAG-Nrf1Δ30 or FLAG-Nrf2 expression plasmids. Although the abundance of FLAG-Nrf1Δ30 and FLAG-Nrf2 was found to be comparable (Fig. 6*A*), the *MT1* reporter gene was strongly activated by FLAG-Nrf1Δ30 but was only weakly activated by FLAG-Nrf2 (Fig. 6*B*). Both Nrf1- and Nrf2-mediated activations were severely compromised when the *MT1* ARE sequence was deleted from the reporter gene, demonstrating that transactivation activity is strictly dependent on the ARE. In stark contrast, FLAG-Nrf2 activated a luciferase reporter gene driven by *NQO1* ARE much more strongly than did FLAG-Nrf1Δ30 (Fig. 6*C*). These results, thus, indicate that the *MT1*

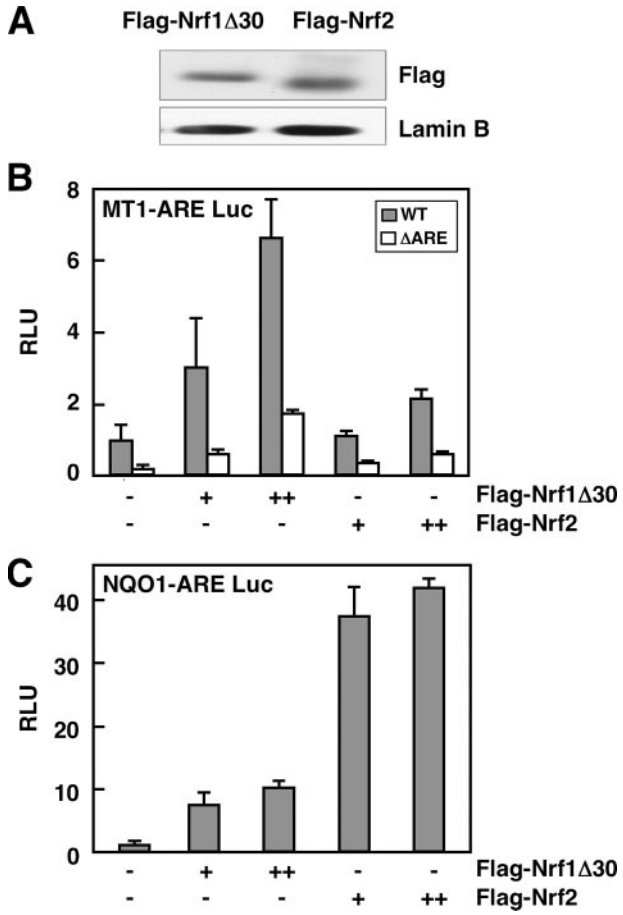

FIGURE 6. **Transactivation activity of Nrf1 and Nrf2 through *NQO1*-ARE and *MT1*-ARE in Hepa1 cells.** *A*, transient overexpression of FLAG-Nrf1Δ30 and FLAG-Nrf2 in Hepa1 cells. Nuclear lysates were examined by immunoblot analysis using an anti-FLAG antibody. Lamin B was detected as a control for nuclear lysate preparation. *B* and *C*, transcriptional activation via *MT1*-ARE and *NQO1*-ARE. Increasing amounts of FLAG-Nrf1Δ30 and FLAG-Nrf2 expression vectors were introduced into Hepa1 cells with pMT1-ARE-Luc (*B*) and pNQO1-ARE-Luc (*C*) reporter constructs. The average values are shown with S.D. The *vertical axis* indicates the relative luciferase unit (*RLU*). Firefly luciferase activity in the absence of effector plasmids is set to 1. *WT*, wild type.

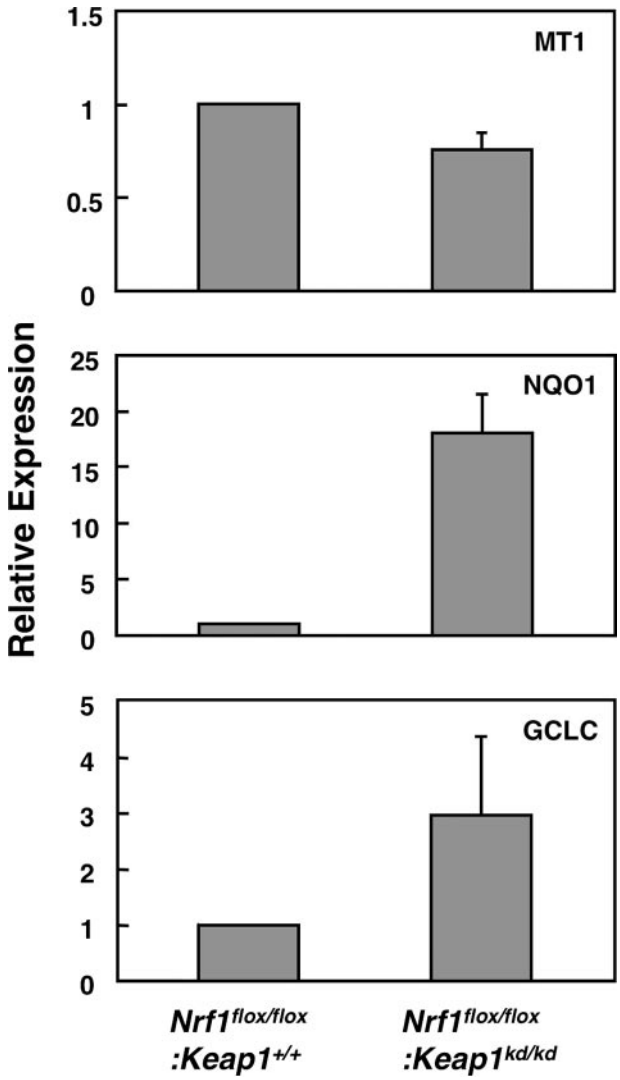

FIGURE 7. **MT1 gene is not activated in the absence of Keap1.** The expression of *MT1*, *NQO1* and *GCLC* genes were examined by quantitative RT-PCR. RNA samples were prepared from *Nrf1*$^{flox/flox}$:*Keap1*$^{+/+}$ and *Nrf1*$^{flox/flox}$:*Keap1*$^{kd/kd}$ mice. The expression level of each mRNA was normalized to the rRNA abundance. The expression of each gene in *Nrf1*$^{flox/flox}$:*Keap1*$^{+/+}$ mouse is set to 1. The average values of are shown with S.D.

gene is an ARE-dependent gene regulated by Nrf1 but not by Nrf2.

MT1 Is Not induced by Constitutive Activation of Nrf2 in Keap1 Knockdown Mutant Mice Liver—To further support our contention that Nrf2 does not regulate *MT1*, we explored whether or not *MT1* is induced in the livers of *Keap1* knockdown mutant mice (*Keap1*$^{kd/kd}$) where Nrf2 can constitutively activate its target genes. Indeed, Nrf2 target genes were induced in *Nrf1*$^{flox/flox}$:*Keap1*$^{kd/kd}$ mice, as has been reported (Fig. 7) (15). No significant difference was observed in the *MT1* gene expression between *Nrf1*$^{flox/flox}$:*Keap1*$^{kd/kd}$ and control *Nrf1*$^{flox/flox}$ mice, conclusively demonstrating that the *MT1* gene is not induced by Nrf2 *in vivo* (Fig. 7).

DISCUSSION

Because Nrf1 and Nrf2 show similar binding specificity and expression profile, these two factors have been suggested to share mutually overlapping target genes and overlapping activities (1, 2). However, gene-targeting experiments in mice revealed that *Nrf1*-null mice (7) exhibit a different phenotype from that of the *Nrf2*-null mice (4), suggesting that these two transcription factors perform distinct functions. However, the analysis of Nrf1 has been hampered because simple knock-out of the *Nrf1* gene in mice provokes embryonic lethality. Therefore, to identify specific roles that Nrf1 plays, we have undertaken a comprehensive exploration of its function *in vivo*. To this end, we generated hepatocyte-specific *Nrf1* gene knock-out mice and examined the pathological and biochemical changes in the livers of the mutant mice. Surprisingly, microarray analyses revealed that loss of Nrf1 did not result in the down-regulation of well known Nrf2-target genes, but rather, Nrf2-target genes were up-regulated in Nrf1 cKO mice. Furthermore, we found that Nrf1 governs stress response genes, such as growth arrest and DNA damage-inducible genes as well as genes involved in glycosylation and the transmembrane transport of xenobiotics. These results, thus, demonstrate that Nrf1 has acquired unique functions that cannot be compensated by the

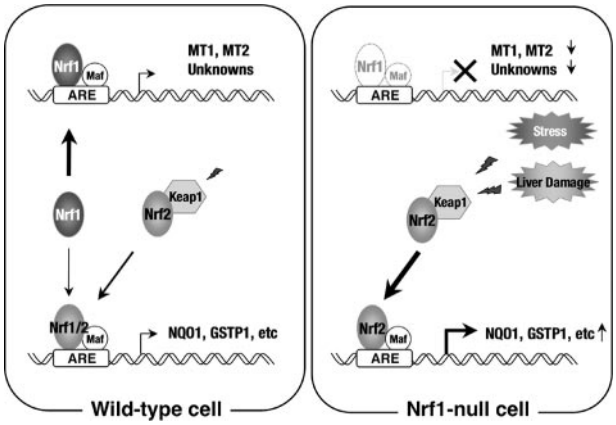

FIGURE 8. **Schematic illustration of differential regulation of ARE-dependent genes by Nrf1 and Nrf2.** In wild-type cells Nrf1 regulates a unique battery of ARE-dependent genes such as *MTs* (see Fig. 4) in addition to typical Nrf2 target genes such as *NQO1* and *GSTP1* according to Leung et al. (8). In the absence of Nrf1, the expression of the latter genes is increased through up-regulation of Nrf2 in response to endogenous stress, presumably due to reduced expression of the former genes.

other CNC family transcription factors during its molecular evolution.

An unexpected finding during the present study was that expression levels of Nrf2-target genes were up-regulated in *Nrf1*$^{flox/flox}$*:Alb-Cre* mice when compared with their expression in control mice. This induction is apparently dependent on Nrf2 as the induction of these genes in *Nrf1*$^{flox/flox}$*:Alb-Cre* mice was reduced upon simultaneous deletion of Nrf2. We envisage that in the absence of Nrf1, Nrf2 can be activated by certain endogenous stresses (Fig. 8). It is well known that Nrf2 is activated by reactive oxygen species, inflammatory cytokines, and ER stresses (26–28). Therefore, a fascinating hypothesis is that the disruption of Nrf1 may provoke ER stress, which eventually leads to the activation of Nrf2 (13, 14).

Consistent with the notion that endogenous stresses activate Nrf2, we recently observed that Nrf2 target genes are induced in liver specific knock-out of *tRNA*^{Sec} (29). In this case, during translation, tRNA^{Sec} inserts selenocysteine into a number of Se-dependent antioxidant enzymes including glutathione peroxidase and thioredoxin reductase (29). These data show that the Nrf2 gene battery represents a second line of defense that enables cells to cope with unscheduled stress that is principally of an exogenous origin. Thus, Nrf2 is crucial for maintaining cellular homeostasis under severe stress conditions. In contrast, Nrf1 is indispensable for countering steady state stress under normal homeostatic conditions, and the lack of Nrf1 activates Nrf2 as a backup defense against the endogenous stressors that are usually neutralized by Nrf1. We envisage the diversity of CNC members has conferred on higher animals multilayered defenses against the numerous endogenous and environmental stressors they encounter.

In the livers of Nrf1 knock-out mice, two of the genes down-regulated to the greatest extent are *MT1* and *MT2*, whose products are cysteine-rich heavy metal-binding proteins. Both *MT1* and *MT2* genes have been reported to harbor at least one ARE in their promoter proximal regions. These AREs have also been reported to mediate the regulation by bZip proteins Fos and Fra-1 (30) and Nrf2 (31). In contrast, another team of researchers reported that *MT1* expression was not decreased in the livers of *Nrf2* knock-out mice (32). In agreement with the latter observation, we found that in the livers of *Keap1* knockdown mice, *MT1* gene expression is not induced in response to the constitutive activation of Nrf2. The microarray analysis further revealed that in the absence of Nrf1, both *MT1* and *MT2* are down-regulated, whereas canonical Nrf2 target genes are up-regulated in an Nrf2-dependent manner. These results, therefore, indicate that both *MT1* and *MT2* genes are specifically activated by Nrf1.

The reporter cotransfection/transactivation analyses support our conclusion that Nrf1 and Nrf2 differentially regulate members of the ARE gene battery. Nrf2 but not Nrf1 effectively activates the NQO1-ARE luciferase reporter gene expression, whereas Nrf1 but not Nrf2 activates reporter gene expression through the *MT1* ARE. In marked contrast, however, electrophoretic mobility shift assay showed that both Nrf1-small Maf and Nrf2-small Maf heterodimers bind to the *MT1* ARE with similar affinity. Therefore, DNA binding specificity seems not to be able to account for their target gene differences. We surmise that whereas both Nrf1 and Nrf2 bind the *MT1* ARE, only Nrf1 can form a complex that activates transcription of the *MT1* gene. Indeed, in contrast to the high level structural conservation of the DNA binding and dimerization domains, the activation domain is not conserved between Nrf1 and Nrf2. Thus, affinity purification-mass spectrometry analysis of the coactivator complexes of Nrf1 and Nrf2 emerges as a viable strategy to answer this important question.

MTs protect hepatocytes against excess zinc and cadmium, free radicals, and various toxic agents (25). For instance, knock-out of the *MT1* and/or *MT2* gene renders mice sensitive to liver damage by alcohol (34). Furthermore, *MT1* and *MT2* double mutant mice have a tendency to suffer from obesity, and although they show increased hepatic lipid accumulation, no hepatitis was observed in these mutant mice (35). At present, a link between MTs and regulation of cellular energy balance is unclear.

Other Nrf1 target genes responsible for the onset of NASH remain to be clarified. We, therefore, searched for ARE sequences within the 400-bp upstream (*i.e.* promoter proximal region) of those genes listed in Table 2, as many functional AREs have been found in the region. We found 11 genes that have potential ARE sequences in the region but have not been reported to be ARE-dependent. Interestingly, among these genes, pyruvate dehydrogenase kinase isozyme 4 is regulated by peroxisome proliferator-activated receptor α (36). Dehydrogenase kinase isozyme 4 is a key regulatory enzyme involved in the switching of energy source from glucose to fatty acids in response to environmental conditions (33, 37). The expression level of peroxisome proliferator-activated receptor α was comparable in *Nrf1*$^{flox/flox}$*:Alb-Cre* mice with the level of expression in wild-type mice (data not shown). We also found that glycosylation-related genes, such as glycosylphosphatidylinositol anchor attachment protein 1 and phosphatidylinositol glycan class O, and stress response gene *Gadd45*γ harbor one or multiple AREs in their promoter proximal regions. Although it remains to be clarified whether there is a direct link between the reduction of these genes and the NASH-like liver damage in

Nrf1$^{flox/flox}$*:Alb-Cre* mice, this mouse model seems to provide an important clue to our understanding of NASH.

As mentioned above, we have generated hepatocyte-specific *Nrf1* knock-out mice independently from the earlier work of Chan and co-workers (10). We have found that the NASH-like liver pathology and lipid accumulation in the mutant mice are in good agreement with the previous report. Importantly, gene expression profiling has not been reported previously in *Nrf1* knock-out mice. Furthermore, among the genes examined previously, we found a few differences in the two hepatocyte-specific *Nrf1* knock-out mice. We surmise that this could be due to a mouse strain difference, or it may be due to different experimental conditions.

In conclusion, we have demonstrated that Nrf1 contributes to the expression of a subtype of ARE-dependent genes that is clearly distinct from those regulated by Nrf2, indicating that each CNC family member has acquired a specific target gene profile. A better understanding of the individual function of CNC members should provide insight into the orchestrated regulation of the ARE gene battery.

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