Oncogenic activation of Nrf2 by specific knockout of Nrf1α that acts as a dominant tumor repressor

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HIGHLIGHTS
- Opposing and unifying inter-regulatory cross-talks between Nrf1α and Nrf2
- Malignant growth of Nrf1α⁻/⁻-derived tumor is prevented by silencing Nrf2
- Hyper-activation of Nrf2 by Nrf1α⁻/⁻ results from decreased Keap1 and PTEN
- Nrf1α⁺/⁺-tumor is repressed by Nrf2⁻/⁻ΔTA, but unaltered by its active caNrf2ΔN

SUMMARY
Liver-specific knockout of Nrf1 in mice leads to non-alcoholic steatohepatitis with dyslipidemia, and its deterioration results in spontaneous hepatoma, but the underlying mechanism remains elusive. A similar pathological model is herein reconstructed by using human Nrf1α-specific knockout cell lines. We demonstrated that a marked increase of the inflammation marker COX2 in Nrf1α⁻/⁻ cells. Loss of Nrf1α leads to hyperactivation of Nrf2, which results from substantial decreases in both Keap1 and PTEN in Nrf1α⁻/⁻ cells. Further investigation of xenograft mice showed that malignant growth of Nrf1α⁻/⁻-derived tumor is almost abolished by silencing Nrf2, while Nrf1α⁺/⁺-tumor is markedly repressed by inactive Nrf2⁻/⁻ΔTA, but unaffected by a priori constitutive activator of caNrf2ΔN. Mechanistic studies unraveled there exist opposing and unifying inter-regulatory cross-talks between Nrf1 and Nrf2. Collectively, Nrf1α manifests a dominant tumor-suppressive effect by confining Nrf2 oncogenicity, while Nrf2 can directly activate the transcriptional expression of Nrf1 to form a negative feedback loop.

Key words: Nrf1α, Nrf2, PTEN, Keap1, COX2, AP-1, tumor repressor, tumor promoter, regulatory networks.

INTRODUCTION
The steady-state lipid levels are crucial for maintaining cellular and organismal homeostasis, not only in term of energy metabolism, but also to prevent potential cytotoxicity. Conversely, excessive nutrients (and metabolic stress) can culminate in a series of severe diseases such as diabetes, obesity and fatty liver. Notably, non-alcoholic fatty liver disease (NAFLD) affects 25% of global population, up to 80% of obese people having this disease (Corte et al., 2015; Younossi et al., 2018). NAFLD comprises a continuum series of pathological conditions varying in severity of liver injury and exacerbation. Among them, non-alcoholic steatohepatitis (NASH) is defined as a serious process with inflammation and hepatocyte damage, and also hence regarded as a major cause of liver fibrosis, cirrhosis, and even cancer, such as hepatocellular carcinoma (HCC), among those caused by unknown etiologies (Friedman et al., 2018; Michelotti et al., 2013; Rowell and Anstee, 2015; Wong et al., 2018). However, the axiomatic mechanisms underlying development of NASH and malignant transformation into hepatoma remain elusive.

The cumulative evidence obtained from distinct animal models resembling human NASH (Friedman et al., 2018) demonstrates that homeostatic and nutrient-stimulated lipid metabolisms are tightly regulated by multiple layers of diverse signaling to transcription factor networks to monitor precision expression of target genes (Jump et al., 2013; Karagianni and Talianidis, 2015). Among them, SREBP1c (sterol-regulatory element binding protein 1c) is established as a key marker and therapeutic target for hepatosteatosis, because transgenic over-expression of this factor leads to hepatosteatosis, but not hepatoma (Nakayama et al., 2007). Yet, similar hyperactivation of SREBP1c by knockout of GP78, an endoplasmic reticulum (ER) membrane-bound E3 ligase, occurs with age-related obesity, NASH and HCC.
(Zhang et al., 2015a). Conversely, hepatosteatosis is partially mitigated by the deficiency of SREBP1c (Yahagi et al., 2002), but sufficiently ameliorated by blockage of SREBP processing by deletion of SCAP (SREBP cleavage-activating protein) (Moon et al., 2012). These findings indicate an additive involvement of other factors beyond SREBPs in NASH-associated malignant pathology.

Interestingly, spontaneous NASH with massive hepatomegaly and hepatoma results from the hepatocyte-specific knockout of PTEN (phosphatase and tensin homolog, a well-known tumor repressor) in mice (Horie et al., 2004). Loss of PTEN leads to constitutive activation of the phosphatidylinositol 3-kinases (PI3K)-AKT-mTOR pathway so as to augment expression of metabolic genes regulated by SREBP1c and PPARY in cancer proliferative cells (Hollander et al., 2011; Lee et al., 2018; Shimano and Sato, 2017). This process is accompanied by nuclear accumulation of Nrf2 (nuclear factor erythroid 2-like 2, also called NFE2L2) in PTEN-deficient cells (Mitsuishi et al., 2012; Sakamoto et al., 2009). Nrf2 and Nrf1 are two principal members of the cap’n’collar (CNC) basic-region leucine zipper (bZIP) family to transactivate antioxidant response element (ARE)-driven genes involved in detoxification, cytoprotection, metabolism and proliferation. Significantly, aberrant accumulation of Nrf2 and activation of target genes are significantly incremented by simultaneous deletion of PTEN (leading to a GSK3β-directed phosphodegron of Nrf2 targeting this CNC-bZIP protein to the β-TrCP-based E3 ubiquitin ligase Cullin 1-mediated proteasomal degradation) and Keap1 (acting as an adaptor targeting Nrf2 to the Cullin 3-mediated proteasomal degradation), resulting in a deterioration of PTEN-ε leading cancer pathology (Best et al., 2018; Rojo et al., 2014; Taguchi et al., 2014). Conversely, malignant transformation of double PTEN:Keap1 knockout mice is alleviated by additive deletion of Nrf2 (Taguchi et al., 2014), implying Nrf2 promotes carcinogenesis. This is consistent with further observations that activity of Nrf2 is required for oncogenic KRAS-driven tumorigenesis (DeNicola et al., 2011) and its activation by antidiabetic agents accelerates tumor metastasis in xenograft models (Wang et al., 2016). However, non-neoplastic lesions are caused by constitutive active Nrf2 (caNrf2) mutants lacking the Keap1-binding sites in transgenic mice (Schafer et al., 2010; Shanmugam et al., 2017), but their cytoprotection against carcinogenesis is enhanced. Further investigation of a dominant-negative dnNrf2 mutant (also suppresses other CNC-bZIP factors, such as Nrf1) has demonstrated that the basal ARE-driven gene expression, but not their inducible expression, is crucial for anti-tumor chemoprevention against the chemical-induced carcinogenesis (auf dem Keller et al., 2006). Yet, the underlying mechanism by which Nrf2 is determined to exert dual opposing roles in tumor suppression or promotion remains unknown to date.

Significantly, another phenotype of spontaneous NASH and hepatoma is manifested in conditional Nrf1−/− mice, displaying a bulk of lipid drops in the ER with dramatic morphological changes (Ohtsuji et al., 2008; Xu et al., 2005). Global knockout mice of Nrf1−/− die of severe oxidative stress-induced damages and fetal liver hypoplasia during development (Chan et al., 1998; Chen et al., 2003). By contrast, global Nrf2−/− knockout mice are viable and fertile, without any obvious pathological phenotypes occurring during normal growth and development (Itoh et al., 1997). Such facts indicate that Nrf1 is not compensated by Nrf2, although both are widely co-expressed in various tissues and have similar overlapping roles in coordinately regulating ARE-driven genes. Further insights also reveal that Nrf1 exerts unique essential functions, which are distinctive from Nrf2, in maintaining cellular redox, lipid and protein homeostasis, as well as organ integrity, through regulation of distinct target genes (Bugno et al., 2015; Zhang and Xiang, 2016). This is reinforced by further investigation of other organ-specific Nrf1 deficiency or over-activation in mice, which exhibit distinct pathological phenotypes, such as type 2 diabetes, neurodegenerative and cardiovascular disease (Hirotsu et al., 2014; Kobayashi et al., 2011; Lee et al., 2011; Zheng et al., 2015). In addition to functionality of Nrf1 as an indispensable CNC-bZIP transcription factor, it is also identified to act as a directly ER membrane-bound sensor to govern cholesterol homeostasis through the consensus recognition motifs (Widenmaier et al., 2017; Zhang et al., 2014b) and lipid distribution in distinct tissues (Bartelt et al., 2018; Hou et al., 2018). However, it is regrettable to unclearly define which isoforms of Nrf1 are required to execute its unique physio-pathological functions, because almost all isoforms of the factor are disrupted to varying extents in the above-described experimental models.
Upon translation of Nrf1, its N-terminal ER-targeting signal anchor enables the nascent full-length protein Nrf1α to be topologically integrated within and around the membranes, while other domains of the CNC-bZIP protein are partitioned on the luminal or cytoplasmic sides (Zhang et al., 2007b; Zhang et al., 2014b). Subsequently, some luminal-resident domains of Nrf1α are dynamically repositioned across membranes through a p97-driven retrotranslocation pathway (Radhakrishnan et al., 2014; Sha and Goldberg, 2014; Zhang et al., 2015b). In these topovectorial processes of Nrf1α, it is subjected to specific post-translational modifications (e.g. glycosylation, deglycosylation, ubiquitination), and also selective juxtamembrane proteolytic processing of the CNC-bZIP factor so as to yield multiple isoforms with different and opposing activities, during maturation into an activator (Koizumi et al., 2016; Xiang et al., 2018a; Xiang et al., 2018b). In addition, distinct variants of Nrf1, including its long TCF11, short Nrf1β/LCR-F1 and small dominant-negative Nrf1γ/δ, are also generated by alternative translation from various lengths of alternatively-spliced mRNA transcripts (Zhang et al., 2014a). Yet, each Nrf1 isoform-specific physiological function virtually remains obscure.

Notably, specific gene-editing knockout of Nrf1α leads to a significant increase in the malignant proliferation of $Nrf1\alpha^{-/-}$-derived hepatoma and the tumor metastasis to the liver in xenograft model mice (Ren et al., 2016). This work reveals that Nrf1α may act as a tumor suppressor, but the underlying mechanism remains unclear. Therefore, our present work further unravels that Nrf1 and Nrf2 have mutual opposing and unified inter-regulatory cross-talks towards downstream genes. For instance, aberrant hyperactivation of Nrf2 leads to a constitutive increase of its target cycloxygenase-2 (COX2) in $Nrf1\alpha^{-/-}$ cells. Such hyper-activation of Nrf2 by knockout of Nrf1α is accompanied by substantial decreases in both Keap1 and PTEN. The malignant growth of $Nrf1\alpha^{-/-}$-derived tumor is significantly prevented by knockdown of Nrf2, while $Nrf1\alpha^{-/-}$-bearing tumor is also markedly suppressed by knockout of Nrf2, but unaffected by a priori constitutive activator of Nrf2 (i.e. caNrf2-On). Such distinct phenotypes of animal xenograft tumors are determined by differential expression of different subsets of genes regulated by Nrf1α or Nrf2 alone or both. These collective findings demonstrated that Nrf1α manifests as a dominant tumor-suppressor to confine Nrf2 oncogenicity. Conversely, though Nrf2 acts as a tumor promoter, it directly mediates the transcriptional expression of Nrf1 so as to form a negative feedback loop.

**RESULTS**

The human $Nrf1\alpha^{-/-}$ and $Nrf2^{-/-}$-driven cell models are established.

Since the phenotypes of liver-specific $Nrf1^{-/-}$ mice resemble human pathogenesis of hepatic steatosis, NASH and HCC (Ohtsuji et al., 2008; Tsujita et al., 2014; Xu et al., 2005), this is thus inferred available for exploring the underlying mechanisms whereby NASH is transformed for the malignant progression towards hepatoma (Figure 1A). However, it is unknown whether human Nrf1α exerts similar effects to those obtained from the aforementioned mouse models. For this end, a similar pathological model was here recapitulated by genome-editing knockout of $Nrf1\alpha$ from human HepG2 cells, aiming to elucidate the mechanism by which a non-resolving NASH-based inflammation is exacerbated. To achieve the genomic locus-specific knockout of $Nrf1\alpha$, we created a pair of TALEN-directed constructs to yield a specific deletion of Nrf1α-derived isoforms from the single $Nfe2l1$ gene, but with shorter variants $Nrf1\beta$ to $Nrf1\delta$ unaffected (Figures 1B and S1A). In the parallel experiments, another pair of CRISPR/Cas9-mediated constructs were engineered to delete the Nrf2-specific codons (covering its 42-175 amino acids within essential Keap1-binding and most of its transactivation domains) from the $Nef2l2$ gene to yield an inactive mutant $Nrf2^{-/-}$STA (Figures 1D, 1E and S1B). Consequently, two monoclonal hepatoma cell lines of $Nrf1\alpha^{-/-}$ and $Nrf2^{-/-}$-STA were, respectively, established and confirmed to be true by sequencing their genomic DNAs, and Western blotting with specific antibodies (Figure 1, B to E). Further real-time qPCRs with specific primers that recognized distinct nucleotide fragments showed that knockout of $Nrf1\alpha$ substantially abolished expression of total Nrf1 mRNAs in $Nrf1\alpha^{-/-}$ cells (Figure 1B, right panel). Similar results were also obtained from other clones of $Nrf1\alpha^{-/-}$ cell lines (Ren et al., 2016). Notably, $Nrf2^{-/-}$-STA cells gave rise to an inactive mutant lacking nt124-526 of Nrf2, but the expression of its DNA-binding domains (DBD)- containing mRNA
transcripts was unaltered (Figure 1D, right panel). Therefore, the resulting inactive mutant $Nrf2^{\Delta TA}$ polypeptides may still, theoretically, bind Nrf2-target genes, and also circumvent competitive occupancy with other complementary factors, upon loss of the prototypic Nrf2.

Figure 1. Establishment of Nrf1α-specific knockout cell models with the NASH phenotype

(A) Schematic diagrams for the liver-specific $Nrf1^{-/-}$ knockout mice that develop spontaneous non-alcoholic steatohepatitis (NASH) and deteriorate into hepatoma eventually.

(B) Both Western blotting (WB, left) and real-time quantitative PCR (qPCR, right) were employed to identify the protein and mRNA levels of Nrf1 in a monoclonal $Nrf1^{-/-}$ knockout cell line. The data are shown as mean ± SEM (n = 3×3, *p < 0.01).

(C) Sequencing peaks of the genomic DNA fragments across the $Nrf1$-specific knockout site, as indicated by alignment with wild type (WT) standard sequence.
D) Expression of inactive Nrf2 mutant protein and mRNA levels in a monoclonal Nrf2-/-ΔTA cell line was identified by WB (left) and qPCR with distinct primer pairs (right). The data are shown as mean ± SEM (n = 3×3, *p < 0.01; NS = no statistical difference).

E) Sequencing peaks of the genomic DNA fragments across the Nrf2-specific knockout site, as indicated by alignment with normal WT sequence.

F) ROS staining of Nrf1/2/-, Nrf1α/- and Nrf2-/-ΔTA cells. They had been treated with 5 μM of DCFH-DA (2',7'-Dichlorodihydro-fluorescein diacetate) for 30 min, before being photographed under a fluorescence microscope. Scale bar = 100 μm.

G) Lipid staining of Nrf1/2/-, Nrf1α/- and Nrf2-/-ΔTA cells, that were or were not treated with 200 μM oleic acid (OA), before being stained with the oil red O agent, and photographed under a microscope. Scale bar = 25 μm.

H) Statistical analysis of the above lipid-stained (G) intensity, that was quantified and shown graphically. The data are represented as mean ± SEM (n = 3), with significant increases ($) or decreases (*), p < 0.01, compared with wild-type values.

I) The expression of inflammation-related genes in Nrf1/2/-, Nrf1α/- and Nrf2-/-ΔTA cells. The data obtained from transcriptome FPKM are shown as mean ± SEM (n=3; $, p < 0.01; $$, p<0.001 and *p < 0.01, by comparison with wild-type).

J) Diagrammatic representation of a proposed model for Nrf1 and Nrf2 to regulate COX1 and COX2 essential for arachidonic acid metabolism relevant inflammatory response.

A plausible explanation of NASH pathogenesis is preferred to the classic two-hit hypothesis, in which the first hit is hepatosteatosis (caused by the accumulation of cholesterol and lipids), and the second hit is inflammation (induced by oxidative stress) (Friedman et al., 2018; Shimano and Sato, 2017). Such being the case, we next examine whether Nrf1α/- cells act accordingly. As anticipated, it is illustrated by measuring intracellular hydrogen peroxide as a representative of reactive oxygen species (ROS), that endogenous oxidative stress was strikingly induced in Nrf1α/- cells, but slightly relieved by inactive Nrf2-/-ΔTA, when compared with wild-type Nrf1/2/-ΔTA progenitor cells (Figure 1F). Subsequently, a significant accumulation of lipids was seen after staining of Nrf1α/- cells, by comparison with Nrf2-/-ΔTA and Nrf1/2/-ΔTA cells (Figure 1G). With increasing time of oleic acid (OA) treatment to 7 days, the lipid accumulation was significantly incremented in Nrf1α/- cells to a maximum ~182-fold estimated. While compared with ~60-fold accumulation of lipids in Nrf1/2/-ΔTA cells, the overload was substantially alleviated by inactive Nrf2-/-ΔTA to ~34-fold (Figure 1, G & H).

In addition to lipid metabolic disorders resulting from loss of Nrf1’s function to regulate LPIN1, PGC-1β and other metabolic genes (Hirotsu et al., 2012; Tsujita et al., 2014), NASH has a not-yet-identified characteristic of refractory inflammation. Accordingly, we determined the transcriptional expression of key genes encoding cytokines and relevant receptors involved in putative inflammatory responses. As expected, expression of all nine examined genes encoding IL-1A, IL-1B, IL-1R1, IL-1R2, IL-6, IL-8, IL-10, TGF1α, and TGF1β was significantly elevated in Nrf1α/- cells (Figure 1I). By contrast with Nrf1/2/-ΔTA cells, mRNA expression of IL-1A, IL-1R2, IL-6 and IL-8 was markedly down-regulated by inactive Nrf2-/-ΔTA, while both IL-1B and IL-10 expression was still marginally up-regulated, but with no changes in other genes (Figure 1I). Collectively, these data demonstrate that the NASH-prone phenotypes are recapitulated by employing human Nrf1α/--driven cells, in which Nrf2 may also be critical for this pathogenesis.

**The inflammation marker COX2 is up-regulated, while COX1 is down-regulated, in Nrf1α/- cells.**

Development of inflammation (e.g. NASH) and malignant transformation into carcinogenesis have a clear relevance to lipid peroxidation, and particularly degradation metabolites of arachidonic acid (AA), such as prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs) (Castellone et al., 2005; Chowdhry et al., 2010; Gupta and Dubois, 2001). In the AA metabolism network, cyclooxygenase 1 (COX1) and COX2 are the rate-limiting enzymes that convert AA into PGs, of which COX2 is considered as a key inflammation marker (Wang and Dubois, 2010) and also identified as a
direct target of Nrf2 (Itoh et al., 2004; Sherratt et al., 2003). Since Nrf2 (and Nrf1) can be recruited to directly bind the ARE-containing promoters of COX2 and COX1 before transactivating both genes, it is thereby hypothesized that hyperexpression of the inflammation-related genes in Nrf1α/− cells is attributable to overstimulation of PG and TX products from the catalyzation by COX2 and COX1 (Figure 1J). To address this, we here examined whether (and how) key rate-limiting enzymes in AA metabolism are influenced by loss of Nrf1α or Nrf2 functions.

As anticipated, a real-time qPCR analysis revealed that mRNA levels of COX1 were almost completely abolished in Nrf1α/− cells, but obviously increased in Nrf2−/−TA cells by comparison to those obtained from wild-type Nrf1/2+/+ cells (Figure 2A). Contrarily, expression of COX2 was substantially augmented in Nrf1α/− cells, but almost unaffected by inactive Nrf2−/−TA when compared to the value in Nrf1/2+/+ cells. Furthermore, expression of ALOX5 and FLAP in Nrf1α/− cells was up-regulated at much higher levels than those measured in Nrf2−/−TA cells at considerable levels (Figure 2A). Next, whether such differences in expression of the AA metabolism genes are attributable to differential and even opposing regulation by Nrf1 and Nrf2 was further examined. Consistently, almost no protein expression of COX1 was detected in Nrf1α/− cells, while the abundances of COX2 and ALOX5 proteins were significantly increased, when compared with Nrf1/2+/+ cells (Figure 2B). However, COX1 was highly expressed in Nrf2−/−TA cells at a greater level than that obtained from wild-type cells (Figure 2C). Conversely, COX2 was substantially diminished or abolished by the inactive mutant Nrf2−/−TA, whereas ALOX5 was almost unaffected (Figure 2C).

Since COX1 is constitutive essential for normal physiological homeostasis, while COX2 is an inducibly expressed enzyme to be stimulated by inflammatory stress (O’Banion, 1999; Tanabe and Tohnai, 2002), the changing trends of COX2 induction by 12-O-Tetradecanoylphorbol-13-acetate (TPA) stimulation are evaluated. As a result, stimulation of Nrf1/2+/+ cells by TPA caused an obvious induction of COX2 expression to a ~14-fold maximum at 2 h after treatment; this value was being maintained to 4 h, before being gradually decreased to a ~5-fold level by 10-h treatment (Figure 2D, 2E). In Nrf1α/− cells, the constitute up-expression of COX2 was set to 18-fold as its starting point, subsequent incremental abundances of this enzymatic protein were further induced to a maximum of ~190-fold by 10-h TPA treatment and maintained before the experiment was terminated (Figure 2E, red curve). Relatively, a weak response of COX2 to TPA stimulation of Nrf2−/−TA cells was also observed from 2 h to 8 h, with an inducible peak at 4 h after treatment (Figure 2E, blue curve). Further assays of the luciferase reporter PCOX2-Luc (in which the 2078-bp promoter COX2 gene was constructed) revealed that transcriptional expression of the reporter gene was induced at 4 h after TPA treatment of Nrf1α/− cells, and the TPA-stimulated increases were continuously maintained until 24 h (Figure S2A, S2B). However, no obvious changes in the PCOX2-Luc activity were detected in TPA-treated Nrf2−/−TA or Nrf1/2+/+ cells. These collective findings imply a striking disparity in Nrf1α- and Nrf2-mediated induction of COX2 by TPA.
Figure 2. Differential or opposing roles of Nrf1α and Nrf2 in regulating COX2 and COX1 genes

(A) The mRNA levels of COX1, COX2, ALOX5, and FLAP was determined by real-time qPCR in Nrf1/2+/+, Nrf1α−/− and Nrf2−/−ΔTA cells. The data are shown as mean ± SEM (n = 3×3, *p < 0.01; $, p < 0.01; $$, p < 0.001).

(B) The protein levels of COX1, COX2, ALOX5, Nrf1, and β-actin (as a loading control) in Nrf1−/− and Nrf1/2+/+ cells were visualized by Western blotting.

(C) Western blotting of Nrf1α−/− and Nrf2−/−ΔTA cells to determine protein levels of COX1, COX2, ALOX5, Nrf1, and β-actin.

(D) Time-course analysis of COX2 in Nrf1/2+/+, Nrf1α−/− and Nrf2−/−ΔTA cells, that had been treated for 1 h to 12 h with...
100 nM TPA (12-0-tetradecanoyl phorbol-13-acetate), before being examined by Western blotting.

(E) The intensity of the above anti-COX2 immunobLOTS (D) was quantified by normalizing the untreated value, which is shown graphically. The data are shown as mean ± SEM (n = 3, *p < 0.01; $, p < 0.01).

(F) After the restoration of Nrf1 into Nrf1α−/− cells by packaging with the Lentivirus, changed abundances of Nrf1, COX1, COX2, and ALOX5 among Nrf1/2+/−, Nrf1α−/− and Nrf1α−/+Nrf1-restored cell lines were examined by Western blotting.

(G) Differences in Nrf2, p-JNK and JNK expression between Nrf1/2+/− and Nrf1α−/− cells were unraveled by Western blotting.

(H) The changes in p-JNK, Nrf2 and COX2 were examined, following 24 h treatment of Nrf1α−/− cells with 20 μM of SP600125.

(I) Alterations in Nrf2, COX2, Fra1, and Jun by siRNA interference with Nrf2 in Nrf1α−/− cells were determined by Western blotting.

(J) The human COX2 promoter-driven reporter Pcox2-luc and its mutant (upper) were constructed before the luciferase assay. Nrf1/2+/− cells were co-transfected with either Pcox2-luc or mutant, together with an internal control reporter pRL-TK, plus an expression construct for Nrf1 or Nrf2 or empty pcDNA3 plasmid and allowed for 24 h recovery before the Pcox2-luc activity was calculated (lower). The data are shown as mean ± SEM (n = 3×3, * p < 0.01; $, p > 0.01 compared to the pcDNA3 values).

Hyper-expression of COX2 results from increased Nrf2 and JNK-mediated AP-1 in Nrf1α−/− cells.

Intriguingly, the abundance of COX2, as a well-known direct target of Nrf2, was not decreased, but rather marginally increased by ectopic expression of Nrf1 that had been restored into Nrf1α−/− cells (Figure 2F), in which expression of COS-1 was also rescued, albeit both genes encompass the ARE sites recognized by Nrf1 and Nrf2 (Itch et al., 2004; Sherratt et al., 2003). These seemingly paradoxical results, along with the above-described data from Nrf1α−/− cells, suggest that Nrf1α may have an ambivalent relationship with Nrf2 in regulating COX1 and COX2 genes. Rather, this confusing but exciting finding arouses our de facto curiosity to explore which possible pathways enable Nrf1 to indirectly regulate COX2 (Figure S3A), albeit this enzyme has been shown to be monitored by CREB, NF-κB, STAT1, FOXM1, ETS1, ELF3 and JNK-regulated AP1 (Ghosh et al., 2007; Grall et al., 2005; Kang et al., 2006; Sharma-Walia et al., 2010; Xu and Shu, 2013; Zhang et al., 2007a). Consequently, the real-time qPCR analysis revealed that mRNA levels of only RELB, but not other members of the NF-κB family (that regulates cellular responses to inflammation), were significantly up-regulated in Nrf1α−/− cells (Figure S3B). This may be coincident with the notion that ablation of an IκB (inhibitor of NF-κB) kinase IKKγ in liver parenchymal cells causes spontaneous NASH and HCC (Luedde et al., 2007). However, Figure S3C showed that the abundance of over-expressed COS2 in Nnf1α−/− cells was unaltered by the caffeic acid phenethyl ester (CAPE, a potent specific inhibitor of NF-κB (Natarajan et al., 1996)), and was also not significantly diminished by JSH-23, a broad spectrum inhibitor of NF-κB (Shin et al., 2004). Thus, it is inefarable there implicates an NF-κB-independent pathway to up-regulate expression of COX2 in Nrf1α−/− cells. In addition, it may be not necessary for modest inducible expression of ETS1 (one of the E26 transformation-specific transcription factors), because this was accompanied by substantial down-regulation of another family member ELF3 (Figure S3B).

Further treatments of Nrf1α−/− cells with either of the two CREB inhibitors H-89 and BAPTA-AM (Sharma-Walia et al., 2010; Tymianski et al., 1994) demonstrated that the elevated expression of COX2 was unaffected (Figure S3D). However, it is, to our surprise (Figure S3E), found that the forced abundance of COX2 in Nrf1α−/− cells was sufficiently abolished by a JNK-specific inhibitor SP600125 (Bennett et al., 2001). Further investigations revealed no changes in both mRNA and protein levels of JNK (Figures 2G and S3), but the phosphorylated JNK abundance was significantly increased in Nrf1α−/− cells when compared to those obtained from Nrf1/2+/− cells (Figure 2G). Therefore, it is initially postulated that Nrf1α−/− cells gave rise to the forced expression of COX2 possibly by activation of JNK signaling. Next, in-depth insights into the signaling of JNK towards downstream target genes unraveled that expression of only c-Jun,
but not other examined genes, was significantly elevated in Nrf1α−/− cells (Figures S4B). Further assays of luciferase reporter genes P_COX2-Luc and P_CoMTP-Luc (in which TRE indicates TPA-responsive element inserted within the promoter region) verified that AP-1 (a functional heterodimer of Jun and Fos) is favorably required for the transactivation of COX2 in Nrf1α−/− cells (Figure S4C). By defining distinct AP-1 components (e.g. Jun, Fos, Fra1) at mRNA and protein levels, it is validated that AP-1 was activated in Nrf1α−/− cells, but not in Nrf2−/−ΔTA cells (Figure S4, D to F). In addition, Figure S4G illustrated that hyper-expression of COX2 in Nrf1α−/− cells was not suppressed by AP-1 inhibitor SR11302 (Fanjul et al., 1994). Further knockdown of Jun or Fra1 led to a decrease of COX2, but this was no proportional to the silencing of Jun or Fra1 at lower levels (Figure S4H). All together, AP-1 activation by JNK signaling is involved in, but not essential for making a significant contribution to the reinforced expression of COX2 in Nrf1α−/− cells.

Fortunately, the evidence that expression of Nrf2 and its nuclear translocation are attenuated by JNK inhibitor SP600125 (Ahn et al., 2017; Bak et al., 2016) implicates there exists a direct link between JNK and Nrf2. Consistently, the abundance of Nrf2 protein was surprisingly augmented in Nrf1α−/− cells, which was accompanied by an increase in the phosphorylated JNK (Figure 2G). Similarly, great increases in expression of both COX2 and Nrf2 were caused by knockout of Nrf1α in HL7702 Nrf1α−/− (established on the base of the non-cancerous HL7702 hepatocyte line (Figure S5A,B). Further treatments of Nrf1α−/− cells with SP600125 or transfection with Nrf2-targeting siRNA, unraveled that reduction of Nrf2 or JNK appeared to be proportional to the decreased abundance of COX2 (Figure 2H, 2I and S4I). Collectively, these data indicate that the hyper-expression of COX2 in Nrf1α−/− cells is directly caused by increased Nrf2 protein, and the latter CNC-bZIP is also monitored by its upstream JNK signaling. This is supported by the P_COX2-Luc reporter assays showing that Nrf2 mediated transactivation of the COX2 gene driven by its ARE enhancer, because the activation was attenuated by its ARE mutant (i.e. P_CoMTP-Luc) (Figure 2J). The transactivation of P_COX2-Luc reporter by Nrf1 (Figure 2J), together with above-described data, indicates that Nrf1α also has one hand to exert a minor positive effect on COX2 by directly binding to its ARE enhancer, but this effect appears to be counteracted by another hand of Nrf1α to elicit a dominant-negative role by indirect inhibitory pathways.

Some AP-1 abundances were obviously suppressed by silencing Nrf2 in Nrf1α−/− cells (Figure 2I), and strikingly prevented by inactive Nrf2−/−ΔTA, by comparison with equivalent controls (Figure S4F). Together with the above-described results, these imply that AP-1 is dominantly repressed by Nrf1α, but positively regulated by Nrf2. Rather, no available evidence has been presented here to support the notion that AP-1 activates transcription of Nrf2 (Tao et al., 2014). In mouse embryonic fibroblasts (MEFs), COX2 is co-regulated by Nrf1 and Nrf2, because its abundance was significantly abolished by global knockout of Nrf1 or Nrf2 (Figure S5C). Here, it should also be noted that global knockout of all mouse Nrf1 or Nrf2 DNA-binding domain-containing fragments was achieved by both gene-targeting manipulations (Ohtsujii et al., 2008; Tsujita et al., 2014). This is totally distinctive from specific gene-editing to delete the designed portions of human Nrf1α or Nrf2 (Figure 1). Importantly, knockout of Keap1 in MEFs (Figure S5D) and human HepG2 (Figure S5E) caused a remarkable increase in expression of Nrf1, Nrf2, COX2, and HO-1 to different extents detected. Overall, the precision regulation of COX2 by Nrf1 and Nrf2, along with Keap1, in distinct manners, is preferable to depend on distinctive cell types in different species.

**Nrf1α and Nrf2 transactivate ARE-driven miR-22 signaling to PTEN, but not to COX1.**

On the contrary to COX2, the isoenzyme COX1 was highly expressed in Nrf2−/−ΔTA cells (Figure 2C), but its expression was almost completely abolished in Nrf1α−/− cells (Figure 2B) and also not rescued by restoration of ectopic Nrf1 into Nrf1α−/− cells (Figure 2F), albeit Nrf2 was up-regulated (Figure 2G). Thereby, it is inferable that no matter how Nrf1α and Nrf2 have opposing or overlapping roles in regulating COX1 expression, Nrf2 exerts a dominant inhibitory effect on COX1, but this effect is fully contrary to regulating COX2. Thus, we speculate that the putative inhibition of COX1 by Nrf2 (and possibly Nrf1α) may be achieved through an indirect miRNA-regulatory pathway, except for directly ARE-
binding to this gene. Fortunately, a candidate miR-22 was selected by predicting possibly miRNA-binding sites within the COX1 3′-UTR region (see http://www.targetscan.org/vert_72/). As expected, the real-time qPCR analysis unraveled that miR-22 expression was significantly increased in Nrf1α−/− cells but decreased in Nrf2−/−ΔTA cells (Figure 3A). Forced expression of Nrf1 or Nrf2 also caused an obvious increase in miR-22 expression in wild-type Nrf1/2+/+ cells (Figure 3B). Further analysis of the miR-22-coding gene revealed there exists a consensus ARE site within its promoter (Figure 3C, upper panel). The promoter-driven luciferase reporter (i.e. miR22-ARE-Luc) was created, so as to assay for its transcription activity. The results showed that the miR22-ARE-Luc reporter gene was significantly transactivated by Nrf1 and Nrf2 (Figure 3C), and the transactivation was diminished by the mutant miR22-AREm-luc. Together, these imply direct and indirect transactivation of miR-22 possibly by Nrf1α and Nrf2.

Figure 3

(A) The content of miR-22 in Nrf1/2+/+, Nrf1α−/− and Nrf2−/−ΔTA cells was determined by qPCR with miR-22 specific primers. The data are shown as mean ± SEM (n = 3×3; *p < 0.01; $, p < 0.01 compared to wild-type values).
(B) The miR-22 expression was altered by transfection of an expression construct for Nrf1 or Nrf2, or an empty pcDNA3 control, into Nrf1/2+/- cells. The qPCR data are shown as mean ± SEM (n = 3×3; $, p < 0.01).

(C) The miR22-ARE-luc reporter driven by an ARE enhancer in the mir-22 gene promoter, and its mutant reporter miR22-AREm-luc were constructed. Either of reporter genes, together with pRL-TK, plus each of pcDNA3, Nrf1, or Nrf2 expression constructs were co-transfected into Nrf1/2+/- cells and then allowed for 24-h recovery before the luciferase activity measured. The data are shown as mean ± SEM (n = 3×3; *p< 0.01; $, p< 0.01)

(D) There exists a miR-22 binding site in the PTEN’s 3’UTR region (that was constructed in the dual fluorescent psiCHECK2 vector to yield the PTEN-miR22b reporter). Either of PTEN-miR22b and PTEN-miR22b-mut was co-transfected with miR-22 expression plasmid or a negative control (NC) into Nrf1/2+/- cells, and then allowed for 24-h recovery, before the fluorescent activity was determined. The data are shown as mean ± SEM (n = 3×3; *p< 0.01; NS= no statistical difference).

(E) Either PTEN-miR22b or PTEN-miR22b-mut was co-transfected with each of pcDNA3, Nrf1, or Nrf2 expression constructs Nrf1/2+/- cells and allowed for 24-h recovery, before the fluorescent activity was measured. The data are shown as mean ± SEM (n = 3×3; *p< 0.01).

(F) The mRNA (upper) and protein (lower) levels of PTEN in Nrf1/2+/-, Nrf1α+/- and Nrf2+/- cells were determined by qPCR and Western blotting, respectively. The data are shown as mean ± SEM (n = 3×3; *p< 0.01; $, p< 0.01).

(G) Schematic representation of the PTEN promoter-containing pPTEN-luc, its distinct ARE-driven reporters (PARE-luc and PARE2-luc) and indicated mutants, which were constructed into the PGL3-Promoter (PGL3-Pro) vector.

(H) The pPTEN-luc and pRL-TK, along with an expression construct for Nrf1 or Nrf2, or pcDNA3 were co-transfected into Nrf1/2+/- cells and then allowed for 24-h recovery before being measured. The luciferase activity data are shown as mean ± SEM (n = 3×3; $, p< 0.01).

(I) Nrf1/2+/- cells were co-transfected with an indicated luciferase reporter, together with pRL-TK and Nrf1 expression construct or pcDNA3 for 24 h before being determined. The data are shown as mean ± SEM (n = 3×3; $, p< 0.01; *p< 0.01).

Since negative regulation of PTEN by miR-22 had been reported (Fan et al., 2016; Tan et al., 2012), a Renilla reporter gene containing the 3’UTR region of PTEN (i.e. PTEN-miR22b) was here constructed, together with a mutant of miR-22-binding site (i.e. PTEN-miR22b-mut, Figure 3D, upper panel). If miR-22 would bind the 3’-UTR region of PTEN transcripts, the PTEN-miR22b-driven Renilla activity was significantly reduced by miR22 (Figure 3D, lower panel), and also partially decreased by ectopic expression of Nrf1 or Nrf2 (Figure 3E). These negative effects were sufficiently abrogated by PTEN-miR22b-mut. Consistently, both mRNA and protein levels of PTEN were significantly reduced in Nrf1α+/- cells (with hyper-expression of Nrf2) but strikingly increased in Nrf2+/-ΔTA cells (Figure 3F). Notably, such opposing changes in PTEN levels are inversely correlated with the relevant values of miR22 measured in same cell lines (Figure 3A). Thus, transactivation of miR-22 by Nrf1α and Nrf2 leads to putative inhibition of PTEN.

To further determine whether such miR-22 pathway is involved in the regulation of COX1 by Nrf1 and Nrf2, the luciferase reporter gene was constructed by cloning the 3’-UTR sequence of COX1 (i.e. COX1-miR22b), along with a mutant of the putative miR-22 binding site to yield a COX1-miR22b-mut reporter (Figure 6A, upper panel). However, the COX1-miR22b-driven Renilla activity was unaltered by miR-22, Nrf1 and Nrf2, when compared with that of COX1-miR22b-mut (Figure S6A1, S6A2). Another luciferase reporter gene (i.e. P COX1-Luc) was engineered by inserting the 1413-bp COX1 gene promoter, but the P COX1-Luc activity was also unaltered by forced expression of Nrf1 and Nrf2 (Figure 6B1). However, the responsiveness of the P COX1-Luc to TPA was induced (Figure S6B2), albeit it was relatively weak, when compared to the P COX2-Luc (Figure S2A). Intriguingly, the P COX1-Luc activity was modestly mediated by Jun, but almost unaffected by a canonical AP-1 dimer (Figure S6B3). This is consistent with the notion from (Smith et al., 1997), but further insights are required into the mechanisms underlying the regulation of COX1 by Nrf1α and Nrf2.

Nrf1α and Nrf2 have mutual regulatory effects on downstream genes.
Since an unusual increase in Nrf2 protein is accompanied by relative higher levels of ROS in Nrf1Δ/Δ cells (Figures 1F, 2G), it is inferable that Nrf1α-deficient hepatoma cells are growing under severe redox stress conditions redefined at a new higher steady-state level leading Nrf2 to become hyperactive. As anticipated, mRNA expression levels of HO-1, GCLC, GCLM, NQO1 and xCT (they are co-target genes mediated by Nrf1 and Nrf2 (Leung et al., 2003; Ohtsuji et al., 2008; Tsujita et al., 2014) were significantly increased Nrf1Δ/Δ cells (Figure 4A). Meanwhile, a marked decrease in LPIN1, but no significant reduction in PGC-1β (both were identified as Nrf1-specific target genes by (Hirotsu et al., 2012)), was determined by comparison with equivalents of wild-type Nrf1/2Δ/Δ cells. Despite no obvious alterations in mRNA levels of Nrf2 (Figure 4A), Western blotting revealed significant increases in the abundance of Nrf2 protein and typical downstream gene products HO-1, GCLM, NQO1 and HIF1α in Nrf1Δ/Δ cells, by contrast with Nrf1/2Δ/Δ cells (Figure 4B). All four protein levels of HO-1, GCLM, NQO1 and HIF1α were, however, markedly reduced in accordance with knockdown of Nrf2, by siRNA-targeting interference with Nrf1Δ/Δ cells (Figure 3C). Silencing of Nrf2 also led to decreased mRNA expression levels of HO-1, GCLM and xCT (Figure S5F). Conversely, restoration of ectopic Nrf1 expression into Nrf1Δ/Δ cells caused obvious decreases in abundances of Nrf2, HO-1, GCLM and NQO1 to different extents as detected (Figure 4D). Collectively, it is demonstrated that in Nrf1Δ/Δ cells, hyper-active Nrf2 has a potent ability to mediate a subset of their co-target genes. Furthermore, the phosphorylated JNK, but not its total, proteins were markedly decreased, as Nrf2 protein was reduced by ectopic expression of Nrf1 after transfecting into Nrf1Δ/Δ cells (Figure 4D). This finding, together with the evidence that Nrf2 is repressed by JNK inhibitor treatment of Nrf1Δ/Δ cells (Figure 2H), implicates that Nrf2 may govern a not-yet-identified upstream kinase to phosphorylate JNK through a positive feedback loop.

By contrast, inactivation of Nrf2 led to strikingly decreases in both mRNA and protein levels of Nrf1 in Nrf2Δ-STA/Δ-STA cells (Figure 4E, 4F). This was accompanied by significantly diminishments in the expression of their co-regulated downstream genes HO-1, GCLM, NQO1 and HIF1α in Nrf2Δ-STA/Δ-STA cells (Figure 4E, 4F), except with a modest reduction in LPIN1 and PGC-1β mRNA levels. Thereby, such marked decreases in expression of Nrf1, HO-1, GCLM, NQO1 and HIF1α resulting from loss of Nrf2 function demonstrate that Nrf2Δ-STA/Δ-STA cell line could provide a favorite model to determine the changing downstream genes regulated by Nrf1, Nrf2 alone or both. Next, to address this, Nrf2Δ-STA/Δ-STA cells were allowed for the ectopic expression of Nrf1 or Nrf2 in order to estimate specific downstream genes. As expected, it is validated that Nrf1-specific target gene PSMB6 was increased by forced expression of Nrf1, but not Nrf2 allowed for restoration in Nrf2Δ-STA/Δ-STA cells (Figure 4G). Conversely, expression of NQO1 was induced by ectopic Nrf2, rather than Nrf1, after being transfected into Nrf2Δ-STA/Δ-STA cells. This implies that NQO1 is Nrf2-dependent, but insensitive to Nrf1 in Nrf2Δ-STA/Δ-STA cells. In fact, Nrf1 and Nrf2 have overlapping roles in mediating transactivation of HO-1 and GCLM (Figure 4G, 4H). Intriguingly, both CNC-ZIP factors also enhanced expression of COX2, but reduced COX1 expression (Figure 4G, 4H). This seems consistent with additional examinations, revealing that silencing of Nrf2 in Nrf1Δ/Δ cells gave rise to a relative higher expression of COX1, with an accompanying decrease in COX2 (Figure S5F). Such co-inhibition of COX1 by two transcriptional activators Nrf1 and Nrf2 is puzzling, albeit it is known that transcriptional expression of downstream genes is mediated by their functional heterodimers with a partner of small MAF or other bZIP proteins through directly binding the cis-regulatory ARE sites in their promoters (Yamamoto et al., 2018; Zhang and Xiang, 2016). Together with the above data (Figures 2, 3), these collective findings indicate that Nrf1 and Nrf2 may also be two indirect players in the transcriptional regulation of COX1 by an unidentified pathway.

To determine which specific genes are constitutively activated by Nrf2, a dominant constitutive active mutant caNrf2ΔN-expressing cell line was established by the gene-editing to delete the N-terminal Keap1-binding portion of Nrf1 (Figure S5A). The resulting caNrf2ΔN cells indeed gave rise to a higher expression of Nrf2, as well as Nrf1, when compared to wild-type cells (Figures 4I, S5G). Interestingly, expression of COX1 almost disappeared as accompanied by significant increases of COX2 in caNrf2ΔN cells (Figures 4I, S5G). This further supports the above evidence obtained
from inactivation of Nrf1α and Nrf2. Constitutive presence of caNrf2ΔN also led to increases in abundances of both HO-1 and GCLM (Figure 4I), in addition to an enhanced expression of xCT and Lpin1 (Figure S5G). Furthermore, phosphorylated JNK was significantly induced by caNrf2ΔN, with no changes in total JNK protein (Figure 4I), implying there may exist a putative upstream kinase monitored by Nrf2.

Figure 4. Opposing and unified cross-talks between Nrf1α and Nrf2

(A) Real-time qPCR determined the mRNA levels of Nrf2, HO-1, GCLC, GCLM, NQO1, LPIN1, and PGC1β expressed in
transcriptional expression of transactivation by Nrf1 and Nrf2 was made, in order to yield a series of truncated mutants from 5025-bp markedly induced by Nrf1 and Nrf2 (Figure 5D). Although no canonical ARE sequence (5′ to 3′)

together with vitamin C (VC, a dual redox inducer) (Figure 5C). Thereby, a mutual inter-regulatory relationship between Nrf1 and Nrf2 was confirmed by real-time qPCR. The data are shown as mean ± SEM (n = 3×3, *p<0.01).

After Nrf1 was allowed for restoration into Nrf1/2−/− cells, changes in transcript levels of Nrf2, HO-1, GCLM, NQO1, p-JNK and JNK were determined in Nrf1/2−/−, Nrf1α−/− cells and Nrf1α−/−+Nrf1-restored cells (E) Expression of Nrf1, HO-1, GCLC, GCLM, NQO1, LPIN1 and PGCI8 genes in Nrf1/2−/− and Nrf2−/− cells were analyzed by real-time qPCR. The data are shown as mean ± SEM (n = 3×3, *p<0.01).

The protein levels of Nrf1, HO-1, GCLM, NQO1 and HIF1α in Nrf1/2−/− and Nrf2−/− cells were visualized by Western blotting.

To further assess a mutual regulatory relationship between Nrf1 and Nrf2, we herein examined whether one of the endogenous proteins was influenced by another of both proteins that were allowed for ectopic over-expression in wild-type Nrf1/2−/− cells. As shown in Figure 4I, the endogenous Nrf2 protein was obviously decreased by ectopic Nrf1. Consequently, abundances of HO-1 and GCLM were markedly increased, whereas COX2 was weakly enhanced, but COX1 was significantly decreased following over-expression of Nrf1 (Figure 4I). By contrast, over-expression of ectopic Nrf2 caused an enhancement in endogenous Nrf1 (Figure 4K). This was accompanied by striking increases of Nrf1 and Nrf2 transactivate Nrf1/2−/− cells. As shown in Figure 4J, the endogenous Nrf2 protein was obviously decreased by ectopic Nrf1 (Figure 4J). By contrast, over-expression of Nrf1, HO-1, GCLM, NQO1 and HIF1α in Nrf1/2−/− and Nrf2−/− cells were visualized by Western blotting.

By cloning the promoter regions of Nrf1 and Nrf2 genes and evaluated their activity by transfection into HepG2 cells (Figure 5A, 5B). As anticipated, the results showed that both P_{nrf1-luc} and P_{nrf2-luc} reporter genes were significantly induced by thapsigargin (TG, a classic ER stressor), or tert-Butylhydroquinone (tBHQ, a typical oxidative inducer), but not vitamin C (VC, a dual redox inducer) (Figure 5C). Thereby, P_{nrf1-luc} and P_{nrf2-luc} reporters are available to assess transcriptional expression of Nrf1 and Nrf2. Subsequently, co-transfection of expression constructs for Nrf1 or Nrf2, together with P_{luc} reporters, revealed that the transcription of P_{luc}, but not P_{nrf2-luc}, genes was markedly induced by Nrf1 and Nrf2 (Figure 5D). Although no canonical ARE sequence (5′-TGACxxGC-3′) exist in the 5025-bp Nrf1 gene promoter, an attempt to identify which sites are located in the promoter enabling for specific transactivation by Nrf1 and Nrf2 was made, in order to yield a series of truncated mutants from P_{nrf2-luc} (Figures 5A, 5B).
Fortunately, the resulting luciferase assays uncovered that several reporters containing the first exon region of Nrf1 were activated by Nrf1 and Nrf2 possibly through different regulatory sites (Figure 5B). From various lengths of the \( P_{Nrf1}\)-luc and mutants, it is inferable that the \( Nrf1/Nfel1\)-regulatory locus site-1 (i.e. Site-1) specific for Nrf2 is located in a 62-bp range between +572 bp and +634 bp, and the \( Nrf1/Nfel1\)-regulatory locus site-2 (i.e. Site-2) specific for Nrf1 \textit{per se} is situated in another 100-bp range from +1031bp to +1131bp (Figure S7A).

Figure 5. Nrf1\( \alpha \) and Nrf2 have inter-regulatory cross-talks at distinct levels
(A) Schematic representation of both Pnrf1-luc and Pnrf2-luc reporters (driven by the human Nrf1 and Nrf2 gene promoters), along with various lengths of truncated Pnrf1-luc mutants as indicated, which were constructed in the PGL3-Basic vector. There exist two transcriptional starting sites (i.e. TSS1, TSS2) within the Nrf1 gene promoter, which contains two Nrf1/Nef2l1-regulatory locus sites (i.e. Site-1, Site-2, also see Figure S6A).

(B) Each of the Pnrf1-luc and indicated mutants, together with pRL-TK, plus an expression construct for Nrf1 or Nrf2 or pcDNA3, were co-transfected into Nrf1/2−/− cells and allowed for 24-h recovery, before the luciferase activity was measured. The data are shown as mean ± SEM (n = 3×3; $, p< 0.01 compared with the transfection with Pnrf1-luc and pcDNA3).

(C) Nrf1/2−/− cells were co-transfected either Pnrf1-luc or Pnrf2-luc, together with pRL-TK, and allowed for 24-h recovery, before being treated with 50 μM tBHQ (tert-butylhydroquinone), 1 μM TG (thapsigargin) or 200 μM VC (vitamin C) for additional 24h, respectively. The data are shown as mean ± SEM (n = 3×3; $, p< 0.01. NS= no statistical difference).

(D) Either Pnrf1-luc or Pnrf2-luc, plus pRL-TK and an expression construct for Nrf1 or Nrf2 at the concentrations as indicated were co-transfected into Nrf1/2−/− cells and then allowed for 24-h recovery before being determined. The data are shown as mean ± SEM (n = 3×3; *p< 0.01).

(E) The pulse-chase analysis of Nrf2 in Nrf1/2−/− and Nrf1α−/− cells were carried out after treatment of these cells with 50 μg/mL of cycloheximide (CHX) alone or plus 5 μM of proteasomal inhibitor MG132 for various lengths of time as indicated.

(F) The stability of Nrf2 was determined with its half-life in Nrf1/2−/− and Nrf1α−/− cells as treated above (E).

(G) Expression of GSK3β, β-TrCP, p62 and Keap1 at mRNA levels in Nrf1/2−/− and Nrf1α−/− cells were examined. The qPCR data are shown as mean ± SEM (n = 3×3, *p< 0.01).

(H) The protein abundances of Keap1, GSK3β and β-TrCP in Nrf1/2−/− and Nrf1α−/− cells were determined by Western blotting.

(I) The mRNA (upper column) and protein (lower panel) levels of PTEN in Nrf1/2−/−, Nrf1α−/− and caNrf2ΔN cells were determined. The data are shown as mean ± SEM (n = 3×3, *p<0.01, NS= no statistical difference).

(J) Nrf1/2−/− and Nrf1α−/− cells had been treated with 100 nM rapamycin (RAPA) for 24 h, before being visualized by Western blotting to detect the changes of p-S6K1, AKT1, Nrf1, Nrf2, HO-1, and COX2 proteins.

(K) An inter-regulatory model is proposed to explain opposing and unifying cross-talks between Nrf1 and Nrf2 at distinct levels.

**Nrf1α−/−-leading accumulation of Nrf2 results from decreased Keap1.**

The putative inter-regulation between Nrf1α and Nrf2 was further investigated to interpret the rationale underlying an abnormal accumulation of Nrf2 protein with no changes in its mRNA expression in Nrf1α−/− cells (Figure 2G,4A). Based on this finding, combined with the notion that Nrf1, but not Nrf2, acts as a primary transcriptional regulator of 26S proteasomal subunits (Radhakrishnan et al., 2010; Steffen et al., 2010), thereby it is hypothesized that aberrant accumulation of Nrf2 results from loss of Nrf1α’s function leading to an imbalance between Nrf2 protein synthesis and degradation processing. As shown in Figure S7(B, C), total protein ubiquitination was significantly accumulated in Nrf1α−/− cells, but not in Nrf2−/−/caN cells, when compared with wild-type cells. Further analysis of mRNA expression levels revealed that 20 of 36 genes encoding all 26S proteasomal subunits and relevant proteins were significantly reduced by knockout of Nrf1α (with only an exception of PSMB10 enhanced) (Figure S7D). By contrast, no marked changes in transcriptional expression of 31 of the above 36 genes (except that PSMB3, PSMB10, PSMC5, and PSMD3 were reduced and PSME1 increased) were determined in Nrf2−/−/caN cells (Figure S7E). Therefore, such proteasomal dysfunction by loss of Nrf1α may result in an accumulation of Nrf2 by impaired proteasomal degradation pathway.

To address this, Nrf2 protein turnover was further determined by pulse-chase analysis of its half-life in Nrf1α−/− cells (Figure 5E). Surprisingly, it was found that Nrf1α−/− cells gave rise to relatively stable protein of Nrf2 with a prolonged half-life to 2.71 h (±163 min) after treatment with cycloheximide (CHX), an inhibitor of newly-synthesized
polypeptides), and also such longevity of Nrf2 was largely unaffected by the proteasome inhibitor MG132 (Figure 5F, lower panel). As controls, Nrf1+/− cells displayed rapid turnover of Nrf2 with a short half-life of 0.38 h (=23 min) after CHX treatment, and this lifetime was extended to 1.17 h (= 70 min) by MG132 (Figure 5F, upper panel). Overall, the aberrant accumulation of Nrf2 in Nrf1+/− cells results from impaired proteasome-mediated degradation.

Next, several upstream regulators of Nrf2 were examined to determine which pathways are impaired towards its protein turnover in Nrf1+/− cells. Intriguingly, an abundance of Keap1 protein was significantly decreased (Figure 5H), even though its mRNA expression levels were unaltered, along with its turnover regulator p62 was strikingly reduced in Nrf1+/− cells (Figure 5G). Thereby, turnover of Keap1 may also occur through a p62-independent pathway. As such, impairment of Keap1-mediated proteosomal degradation of Nrf2, in particular, oxidative stress (Kobayashi et al., 2006), is likely to contribute to an aberrant accumulation of Nrf2 by the loss of Nrf1α. However, accumulation of Nrf2 is attributable to another impairment of GSK3β-phosphorylated β-TrCP-mediated proteosomal degradation of the CNC-bZIP protein. This is due to a marked decrease of GSK3β at mRNA and protein levels in Nrf1+/− cells (Figure 5G,5H).

**Nrf1α and Nrf2 exert opposing and unifying roles in the regulation of PTEN signaling.**

More importantly, we found that both protein and mRNA levels of PTEN (acts as a key master versatile regulator of Nrf2, Keap1, PI3-kinase, AKT and GSK3β) (Pitha-Rowe et al., 2009; Rojo et al., 2014; Sakamoto et al., 2009; Taguchi et al., 2014), were significantly diminished or even abolished in Nrf1+/− cells (retaining high expression of Nrf2) (Figure 5I, left panel). In contrast, inactivation of Nrf2 caused a striking increase in PTEN mRNA, but not its protein levels, in Nrf2−/−STA cells (yielding low expression of Nrf1) (Figure 3F, 4F). On the contrary, in caNrf2ΔN cells (giving rise to a high expression of Nrf2 and Nrf1, Figure 4I), a significant decrease in PTEN protein abundance, but not its mRNA levels, was determined (Figure 5I). Collectively, together with the above-described data (Figure 3), both Nrf1α and Nrf2 are much likely to exert opposing and unifying roles in the precision regulation of PTEN by both miR-22-dependent and -independent pathways, in which Nrf2 is preferably dominant-negative, whereas Nrf1α has a limited positive role.

Further analysis of the PTEN gene unraveled that there exist two typical ARE sites within its promoter region (Figure 3G). The luciferase assays demonstrated that transcription activity of the PTEN promoter-driven luciferase reporter P<sub>P<sub>PTEN-luc</sub></sub> was significantly induced by Nrf1, but not by Nrf2 (Figure 3H). Mutagenesis analysis uncovered that the second ARE2 site made a primary contribution to transactivation activity of the P<sub>P<sub>PTEN-luc</sub></sub> reporter by Nrf1, while the first ARE1 site also gained a minor contribution to Nrf1-mediated transactivation of P<sub>P<sub>PTEN-luc</sub></sub> (Figure 3I).

Based on the fact that loss of PTEN function leads to constitutive activation of the PI3K-AKT signaling pathway to augment the nuclear accumulation of Nrf2 and its resulting activation (Mitsuishi et al., 2012; Sakamoto et al., 2009), we determined whether the PI3K-AKT signaling is activated by abolishment of PTEN in Nrf1+/− cells (in which Nrf2 is aberrantly accumulated). The results demonstrated Nrf1+/−-leading increased abundances of Nrf2, AKT1, COX2 and HO-1, but such increases were significantly suppressed by the classic mTOR inhibitor, rapamycin (RAPA) (Figure 5J). This implies that the mTOR may also be activated in Nrf1+/− cells. Accordingly, increased abundances of both AKT and phospho-S6K1 in Nrf1+/− cells were markedly blocked by mTOR inhibitor RAPA. This is inversely correlative with the consequence that over-expression of Nrf1 suppresses AKT induction (Hirotsu et al., 2014). Together, this study indicates opposing and unifying cross-talks between Nrf1α and Nrf2 to regulate the PTEN-mTOR-AKT signaling to the Nrf2-COX2 pathway. Based on these findings, we summarized an endogenous inter-regulatory network (Figure 5K).

**Blockage of Nrf1+/−-bearing or Nrf1+/−-derived tumor growth by Nrf2 deficiency.**

Our previous work revealed that malignant growth of Nrf1+/−-derived hepatoma is accompanied by metastasis to the liver in xenograft mice (Ren et al., 2016). Here, to elucidate what effects have been elicited by Nrf1α and Nrf2 on tumor repression or promotion, we further investigate distinct genotypic tumors derived from Nrf1/+/−, Nrf1+/−, Nrf1+/−+siNrf2, Nrf2−/−STA and caNrf2ΔN cells in xenograft mice. Their tumorigenicity was evaluated by measuring
tumor volume and weight. As illustrated in Figure 6(A to C), Nrf2^ΔNΔα^ -cells were inoculated in nude mice, but did not form more than one solid tumor. This implies that tumorigenicity of Nrf1/2^ΔΔα^ -cells, as controls, is almost completely abolished by inactivation of Nrf2. Conversely, constitutive activation of Nrf2 did not obviously influence the resulting coNrf2^ΔΔα^ -driven tumorigenicity, by comparison to that of Nrf1/2^ΔΔα^. This indicates that Nrf2-prone cancer promotion is dominantly confined by the presence of Nrf1α. Upon loss of Nrf1α function in Nrf1α^ΔΔα^ -cells (in which hyper-active Nrf2 is accumulated), the resultant tumorigenicity was hence significantly higher than that of Nrf1/2^ΔΔα^ -tumor, but rather was much strikingly suppressed by silencing of Nrf2 (in the Nrf1α^ΔΔα^-siNrf2-derived tumors) to the much less extent than that of Nrf1/2^ΔΔα^ -cells (Figure 6, A to C). Collectively, these findings demonstrate that Nrf2 acts as a tumor promoter, but it is efficiently confined by Nrf1α serving as a dominant tumor repressor, implying both are a pair of mutual antagonizing twin factors. Overall, malignant transformation of Nrf1α^-ΔΔα^ -derived cells is attributable to hyperactivation of Nrf2.

Histological examination showed that a considerable number of blood vessels were formed in Nrf1α^-ΔΔα^ -tumors but was reduced by Nrf2 knockdown in Nrf1α^-ΔΔα^+siNrf2-derived tumors (Figure 6D). However, no marked differences in vascularity between coNrf2^ΔΔα^- and Nrf1/2^ΔΔα^-bearing tumors were observed. Further insights into angiogenesis-related genes revealed that mRNA expression levels of VEGFA, VEGFC, VEGFD, EGFR, but not HIF1α or STAT1 were strikingly elevated by knockout of Nrf1α, but the increased expression of VEGFC, VEGFD and EGFR was significantly reduced by silencing Nrf2 (Figure 6E). Notably, knockdown of Nrf2 almost completely abolished expression of HIF1α and STAT1 in Nrf1α^-ΔΔα^+siNrf2 cells, but no changes in these two factors were observed in Nrf1α^-ΔΔα^-cells, as compared to those obtained from Nrf1/2^ΔΔα^- cells. Rather, all the other angiogenesis genes except VEGFD were up-regulated in coNrf2^ΔΔα^- cells (giving high expression of Nrf1α and Nrf2), while only STAT1 but not other genes were up-regulated by inactive Nrf2^ΔΔα^ -cells (Figure 6E). Altogether, both Nrf1α and Nrf2 are diversely involved in regulating the expression of angiogenesis genes except for STAT3 as examined above.

Intriguingly, the vascularity of Nrf1α^-ΔΔα^+siNrf2-derived tumors seemed to be higher than that of Nrf1/2^ΔΔα^-bearing tumors (Figure 6D), but, such angiogenic changes cannot explain the observation that the Nrf1α^-ΔΔα^+siNrf2-tumor volumes and weights were significantly less than those obtained from the Nrf1/2^ΔΔα^-tumors. This implicates other rationales beyond angiogenesis. Thus, we employed flow cytometry to determine changes in the cell cycle and apoptosis in five distinct cell lines. As shown in Figure 6(F, G), the S-phase of Nrf1α^-ΔΔα^+siNrf2 cells was significantly shortened. Such a severe S-phase arrest of cell cycle is supported by quantitative gene expression analysis revealing that significant up-regulation of p16, p19, p21 p53 and CDK4 was simultaneously accompanied by down-regulation of RB1, CDK1, CyclinA2, CyclinB2, E2F3, E2F5, and E2F6 in Nrf1α^-ΔΔα^+siNrf2 cells, when compared with its progenitor Nrf1/2^ΔΔα^- or Nrf1α^-ΔΔα^- cells (Figure S8A). In addition to the S-phase arrest, the G0/G1-phase was relatively extended in Nrf1α^-ΔΔα^+siNrf2 cell cycle (Figure 6G). Consistently, apoptosis of Nrf1α^-ΔΔα^+siNrf2 cells was also significantly enhanced, when compared to other cell lines (Figure 6H, SBD). This is also supported by further analysis of apoptosis-related genes unraveling that Box, Bak, Bid, Bad, and Puma were significantly up-regulated, while anti-apoptotic BCL-2 gene was down-regulated, with no changes in BCL-xL and Mcl-1 in Nrf1α^-ΔΔα^+siNrf2 cells (Figure 6I).

Although no significant differences in growth and vascularity of between coNrf2^ΔΔα^- and Nrf1/2^ΔΔα^-bearing tumors, the G2/M-phase of the coNrf2^ΔΔα^- cell cycle was shortened, along with the S-phase extended (Figure 6G). This implies that a G2/M-phase arrest is likely caused by the constitutive activation of Nrf2, in agreement with the supportive evidence that inactivation of Nrf2 markedly prolonged the G2/M-phase of Nrf2^-ΔΔα^- cells (Figure 6G). Further gene expression analysis revealed that p15, p21 and Puma were significantly up-regulated, but p18, CDK1, E2F2 and Bid were down-regulated by coNrf2^ΔΔα^- (Figure 6I, S8A). Conversely, inactive Nrf2^-ΔΔα^- still up-regulated RB1, CDK1, E2F3, and Cyclin D1 (Figure S8A), but strikingly down-regulated FTH1 and FTL (both encode ferritin heavy and light chains involved in iron-dependent lipid peroxidation and ferroptosis, in Figure S8G). Overall, these demonstrate that Nrf1α
and Nrf2 coordinately regulate certain key genes involved in cell cycle and apoptosis.
(E) The qPCR analysis of some angiogenesis-related genes in distinct cells as indicated was validated by transcriptome. The data are shown as mean ± SEM (n=3; *p<0.01; **p<0.001; $, p<0.01; $$, p<0.001).

(F and G) The flow cytometry analysis of distinct cell cycle was indicated. The data (n=3) are shown in two different fashions.

(H) The early apoptosis (EA) and late apoptosis (LA) of five distinct cell lines was examined by flow cytometry. The data are shown as mean ± SEM (n=3; $, p<0.01).

(I) Expression of some apoptosis-related genes in different indicated cells was transcriptomically analyzed. The data are shown as mean ± SEM (n=3; *, p<0.01; ** p<0.001; $, p<0.01; $$, p<0.001).

Different subsets of genes are finely regulated by Nrf1α, Nrf2 alone or both.

Nrf1 and Nrf2 are two important CNC-bZIP transcription factors that are widely expressed in various tissues and also regulate seemingly similar expression patterns of ARE-driven downstream genes that have been identified (Bugno et al., 2015; Tebay et al., 2015). In fact, the ever-accumulating evidence demonstrates that Nrf1 and Nrf2 exert many different and even opposing functions and in particular, unique indispensable functions of Nrf1 are not substituted by Nrf2 (Zhang and Xiang, 2016). Accordingly, the above-described data also unraveled that both CNC-bZIP factors have elicited mutual synergistic and antagonistic roles in regulating precision expression of cognate genes in distinct cell processes, aiming to maintain normal cellular homeostasis. Here, to further evaluate the functional similarities and differences between Nrf1α and Nrf2, the genome-wide expression of genes in Nrf1α−/+, Nrf1α−/−, Nrf1α−/+ siNrf2, Nrf2−/−/STA and caNrf2DN cells was determined by transcriptome sequencing. Those detectable genes with a fold change ≥ 2 and diverge probability ≥ 0.8 were defined as differentially expressed genes (DEGs), by comparison with equivalents measured from Nrf1/2−/+ cells (Figure 7A). Consequently, Nrf1α−/+ cells gave rise to 1,213 of DEGs (i.e. 697 up- plus 850 down-regulated), but the number of DEGs in Nrf1α−/+siNrf2 cells was significantly increased to 3,097 genes, 2247 of which were down-regulated by siNrf2. Intriguingly, only 545 of DEGs were detected in Nrf2−/−/STA cells, implying that many genes are silenced or prevented by inactive Nrf2 mutant (distinctive from simple knockout of this factor). These data suggest that, in this regulatory system by the cooperation of Nrf1 and Nrf2, a single change of both has only limited effects on overall gene expression, and thus both changes will have a greater impact. For instance, when compared to those of Nrf1α−/− cells, silencing of Nrf2 caused 124 genes to be up-regulated, and still led 1,338 genes to be down-regulated in Nrf1α−/+siNrf2 cells (Figure 7A, last column), so that malignant growth of Nrf1α−/−-derived tumor was repressed by Nrf2 knockdown. Conversely, reinforced expression of Nrf2 (and Nrf1) in caNrf2DN cells up-regulated 1655 genes and also down-regulated 423 genes. These findings indicate that Nrf2 is a dominant activator to regulate many genes, while Nrf1α appears to exert dominant negative effects on some genes. Enrichment analysis revealed that DEGs of Nrf1α−/+ cells were subject to 16 pathways (p < 0.001), of which 9 are responsible for human disease and 4 are involved in the environmental information processing (Table S1). By contrast, most of the cellular processes were significantly changed in Nrf2−/−/STA and Nrf1α−/+siNrf2 cells. Thus, loss of Nrf1α relevant to the disease suggests that its function is essential for maintaining cellular homeostasis, while Nrf2 exerts its greater roles in regulating most of cellular physiological processes. For example, the above-described alterations in the cells cycle of Nrf1α−/+siNrf2 were also validated by transcriptome (Table S1). Further calculation of the DEGs distribution unraveled that signal transduction, cancer-relevant, immune system and metabolism were most abundant secondary KEGG pathways in Nrf1− or Nrf2-deficient cells (Figure 7B). An insight into the cellular signaling transduction uncovered that the most DEGs are involved in the PI3K-AKT pathway (Table S1). In this pathway, a key tumor suppressor PTEN was significantly and oppositely altered in both Nrf1α−/− and Nrf2−/−/STA cell lines (Figures 7F, 7C, 7D). Based on these specific findings, much-focused DEGs in Nrf1α−/− and Nrf2−/−/STA cells were mapped according to the KEGG pathway. The results illustrated that both cell lines displayed such significant opposing changes in the PI3K-AKT pathways (Figures 7C, 7D, S9A). As interested, knockout of Nrf1α (with accumulated Nrf2) caused a general reduction...
in transcription of most AKT-signaling molecules, but they were hence generally increased by inactivation of Nrf2. Such striking disparity is dictated by the distinction of Nrf2 proteins in between the two cell lines (Figure 2).

**Figure 7**

(A) Differentially expressed genes (DEGs) in distinct cell lines were analyzed by transcriptome sequencing. The differences in the number of DEGs are shown after being compared with wild-type or indicated cells. Those increased or decreased DEGs were represented by red or green columns, respectively. The DEGs were selected.

**Figure 7. An axiomatic rationale underlying distinct animal xenograft tumor phenotypes.**

(A) Differentially expressed genes (DEGs) in distinct cell lines were analyzed by transcriptome sequencing. The differences in the number of DEGs are shown after being compared with wild-type or indicated cells. Those increased or decreased DEGs were represented by red or green columns, respectively. The DEGs were selected.
genes were up-regulated and 13 genes were down-regulated. Meanwhile, 38 Nrf2-specific downstream genes were regulated specifically. Consequently, 30 Nrf1-specific downstream genes were shown (in Figure 7G, left panel), of which 17 genes were up-regulated and 13 genes were down-regulated. Meanwhile, 38 Nrf2-specific downstream genes were also found herein, of which 25 were up-regulated and 13 were down-regulated (right panel). Collectively, our findings provide an axiomatic rationale for differential expression of different subsets of genes to dictate distinct phenotypes of animal xenograft tumors (Figure 7H). Significantly, the malfunction of Nrf2 is as a potent tumor promoter, but it can be efficiently confined or suppressed by Nrf1α acting as a dominant tumor repressor.

**DISCUSSION**

Accumulating evidence has demonstrated that Nrf1 is a key player in the pathogenesis of NASH and HCC, as well as in cellular signaling cascades, but its role in metabolism appears to be restricted possibly by Nrf1α knockdown (Figure S9B-D). About 24% of these genes are responsible for metabolism-related enzymes. This implies that the function of Nrf2 is closely related to cellular metabolism, particularly in the absence of Nrf1α. This is further evidenced by results from Nrf1α−/−ΔN cells, revealing that many of those accumulated Nrf2α’s effects on downstream genes by Nrf1α−/− were strikingly reduced by knockdown of Nrf2. Therefore, by comparison of the DEGs between Nrf1α−/− and Nrf1α−/−ΔN cell lines, an opposite expression profiling of 87 genes was uncovered by Nrf2 knockdown (Figure S9B-D). About 24% of these genes are responsible for metabolism-related enzymes. This implies that the function of Nrf2 is closely related to cellular metabolism, particularly in the absence of Nrf1α. This is further approved by another opposite expression profiling of other 83 DEGs in between Nrf2−/−ΔTA and caNrf2ΔN cell lines (Figure S10). Still 16% of the differential expression genes are related to metabolism process, but 24% of these genes are involved in signaling transduction. This observation indicates that in the presence of Nrf1α, Nrf2 is a major player in cellular signaling cascades, but its role in metabolism appears to be restricted possibly by Nrf1α.

The Venn diagrams illustrated that distinct subsets of DEGs were regulated by Nrf1, Nrf2 alone or both (Figure 7E). The common genes regulated by Nrf1α and Nrf2 were seen by comparison of DEGs in Nrf1α−/− or caNrf2ΔN with wild-type. In the intersection of Nrf1α−/− and caNrf2ΔN, the remaining portions after excluding Nrf1α−/−ΔN were composed of the (red numbered) genes closely correlated to regulation by Nrf1α. The genes regulated by Nrf2 were also found by comparison of DEGs in Nrf1α−/−ΔN with Nrf1α−/−, as well as Nrf2−/−ΔTA or caNrf2ΔN with wild-type, so the intersection of these three sets comprised the (blue numbered) genes preferably regulated by Nrf2. Further, based on the changes in Nrf1 and Nrf2 proteins detected in distinct cell lines (Figure 7F), we screened which portions of high-relevant downstream genes were consistent with or opposite to the changing trends of Nrf1 or Nrf2, respectively. Consequently, 30 Nrf1α-specific downstream genes were shown (in Figure 7G, left panel), of which 17 genes were up-regulated and 13 genes were down-regulated. Meanwhile, 38 Nrf2-specific downstream genes were also found herein, of which 25 were up-regulated and 13 were down-regulated (right panel). Collectively, our findings provide an axiomatic rationale for differential expression of different subsets of genes to dictate distinct phenotypes of animal xenograft tumors (Figure 7H). Significantly, the malfunction of Nrf2 is as a potent tumor promoter, but it can be efficiently confined or suppressed by Nrf1α acting as a dominant tumor repressor.
other relevant cardiovascular diseases and type 2 diabetes (Bugno et al., 2015; Zhang and Xiang, 2016). However, it should be noted that these experimental mouse genomes were manipulated to delete all Nrf1 isoforms from the single Nrf1/Nef2l1 gene. In this study, human Nrf1α-specific knockout was achieved by the gene-editing to create the frameshift mutation. The phenotypes of NASH and malignancies were reconstructed by using monoclonal Nrf1α−/− cell lines. Thereby, this provides an available model for a follow-up study to elucidate the relevance of Nrf1α with NASH and its malignance into HCC. In the Nrf1α−/−-leading model, the inflammation marker COX2 is constitutively increased, which entails a non-resolving feature. By contrast, the development-related COX1 was almost completely abolished in Nrf1α−/− cells. The resultant metabolites of arachidonic acid by the rate-limiting enzyme COX2, which also serves as a direct target of Nrf2 (Itoh et al., 2004; Sherratt et al., 2003), are much likely to play a crucial role in the development and progression of inflammation, particularly NASH and hepatoma caused by knockout of Nrf1α.

Further examinations revealed that the Nrf1α−/−-caused increase of COX2 occurred by accumulated Nrf2 protein, but both were effectively diminished by inhibitors of JNK (i.e. SP600125) and mTOR (i.e. rapamycin). Hence, the Nrf2-COX2 pathway is inferable to be regulated by both JNK and mTOR signaling, albeit the detailed mechanisms remain unclear. Here, we found that inhibition of the Nrf2-COX2 pathway is accompanied by decreases in AKT, S6K1 and GSK3β. This is consistent with the claim that Nrf2 is regulated by the mTOR-AKT-GSK3β signaling components (i.e. Jun, Fra-1) are mediated by Nrf2 insofar as to form a feedback loop. Contrary to Nrf1α−/−, MEFs of Nrf1−/−(ΔDBD), in which almost all DBD (DNA-binding domain)-containing Nrf1 isoforms are disrupted (Hirotsu et al., 2012; Ohtsuji et al., 2008; Tsujita et al., 2014; Xu et al., 2005), exhibited marked decreases in total COX2 and most of Nrf2 to much lower levels roughly similar to those determined in Nrf2−/−(ΔDBD) MEFs. (Figure S5C). This difference between human Nrf1α−/− and mouse Nrf1−/−(ΔDBD) demonstrates Nrf1 isoform-dependent regulation of the Nrf2-COX2 pathway in distinct species as experimented.

Notably, an accumulation of free radicals in Nrf1−/−(ΔDBD) MEFs results from decreased expression of ARE-driven genes involved in glutathione synthesis, antioxidant and detoxification (Kwong et al., 1999). Similar but different stress caused by liver-specific knockout of Nrf1−/−(ΔDBD) activates a subset of Nrf2-dependent ARE-battery genes in mice, but Nrf2 cannot still compensate for the loss of Nrf1’s function leading to NASH and HCC (Ohtsuji et al., 2008; Xu et al., 2005). The inducible liver-specific knockout of Nrf1−/−(ΔDBD) in mice increased glutathione levels; this results from up-regulation of xCT (a component of the cystine/glutamate antiporter system Xc−), but with no changes in glutathione biosynthesis enzymes (Tsujita et al., 2014). In this work, human Nrf1α−/− increases ROS and lipid levels, along with high expression of xCT and other ARE-driven genes (e.g. HO-1, GCLC, GCLM, NQO1). These genes are Nrf2-dependent because their expression is reduced by inactive Nrf2−/−ΔTA mutant and also repressed by silencing of Nrf2 (in Nrf1α−/−ΔsiNrf2 cells). In addition to COX1 and COX2, both Alox5 and FLAP (also involved in arachidonic acid metabolism) are significantly up-regulated in Nrf1α−/− cells, and also modestly increased in Nrf2−/−ΔTA cells. However, liver-specific Nrf1−/−(ΔDBD) mice display no changes in COX1, COX2 and Alox5 expression (Tsujita et al., 2014). Overall, these discrepancies are likely attributed to the variations of which Nrf1 isoforms have two-sided effects on Nrf2 and other relevant cardiovascular diseases and type 2 diabetes (Bugno et al., 2015; Zhang and Xiang, 2016). However, it should be noted that these experimental mouse genomes were manipulated to delete all Nrf1 isoforms from the single Nrf1/Nef2l1 gene. In this study, human Nrf1α-specific knockout was achieved by the gene-editing to create the frameshift mutation. The phenotypes of NASH and malignancies were reconstructed by using monoclonal Nrf1α−/− cell lines. Thereby, this provides an available model for a follow-up study to elucidate the relevance of Nrf1α with NASH and its malignance into HCC. In the Nrf1α−/−-leading model, the inflammation marker COX2 is constitutively increased, which entails a non-resolving feature. By contrast, the development-related COX1 was almost completely abolished in Nrf1α−/− cells. The resultant metabolites of arachidonic acid by the rate-limiting enzyme COX2, which also serves as a direct target of Nrf2 (Itoh et al., 2004; Sherratt et al., 2003), are much likely to play a crucial role in the development and progression of inflammation, particularly NASH and hepatoma caused by knockout of Nrf1α.

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Albeit Nrf1 and Nrf2 are recruited for directly binding the ARE sites in the COX1 and COX2 promoter regions (Itoh et al., 2004; Sherratt et al., 2003), our evidence unravels that both CNC-bZIP factors have different or opposing roles in bi-directional regulation of COX1 and COX2 by distinct interrelated positive and negative pathways. In particular, Nrf1α has a two-handed potency to execute as an activator or repressor, depending on distinct cognate genes (e.g. COX1 and COX2), through different regulatory pathways. For example, regulation of COX2 is contributed positively by
Nrf2 and negatively by Nrf1α, albeit its promoter-driven Pcox2-Luc reporter is also transactivated by Nrf1 and Nrf2. The direct activation by Nrf1 (as well as Nrf2) may be neutralized or counteracted by its dominant-negative effects triggered by indirect mechanisms (as shown in Figure 5K).

Just contrary to COX2, expression of COX1 is regulated positively by Nrf1α but negatively by Nrf2, albeit no direct activation of its promoter-driven Pcox1-Luc reporter by ectopic Nrf1 and Nrf2 was detected herein. In an attempt to explore into the mechanisms by which COX1 is indirectly regulated by Nrf1/2, we found that both CNC-bZIP factors can directly activate the expression of miR-22 driven by its ARE site. The miR-22, along with Nrf1 and Nrf2, all inhibit the PTEN-miR22b-Renilla reporter activity, implying that these two factors have an intrinsic ability to suppress the tumor suppressor PTEN through activating miR-22, as consistent with the previous reports (Tan et al., 2012; Xu et al., 2012). However, de facto endogenous expression of PTEN at mRNA and protein levels is almost completely abolished in Nrf1α−/− cells (retaining hyper-active Nrf2), but also dramatically increased in Nrf2−/− cells (with decreased Nrf1). Collectively, these findings demonstrate that Nrf2 is a dominant negative to inhibit PTEN; this is further evidenced by a significant reduction of PTEN by a priori constitutive activation of Nrf2 in caNrf2ΔN cells (also with enhanced Nrf1α). By contrast, Nrf1α has Ying-Yang two-sided effects on PTEN. On one side, Nrf1α acts as a major positive regulator of PTEN, while on the other side of Nrf1α, it is enabled to exert a minor negative role in PTEN, but this negation could be concealed by dominant negative Nrf2 or counteracted by the major positive action of Nrf1α per se.

Since PTEN is known to act as the most critical inhibitor of the PI3K-AKT pathway (Tan et al., 2012; Xu et al., 2012), thereby, inactivation of PTEN by ROS provokes activation of its downstream PI3K-AKT signaling cascades to promote cell survival (Chetram et al., 2011; Kitagishi and Matsuda, 2013). Notably, the ever-increasing evidence demonstrates that PTEN can direct inhibition of expression of ARE-driven gene by inhibiting Nrf2 (Rojo et al., 2014; Sakamoto et al., 2009). Taken together with our results from this study, it is demonstrated that the cellular ROS levels are monitored by Nrf1α- and/or Nrf2-mediating ARE-battery genes, but in turn, expression of Nrf1β/2-target genes is also negatively regulated by ROS-activated miR-22-PTEN signaling to form a feedback regulatory circuit. Of note, activation of Nrf1β/2 by ROS can promote miR-22 expression, which may serve as an important approach to regulate the PTEN-PI3K-AKT pathway. Thereby, the quantitative regulations of cellular ROS levels are achieved by close cooperation of Nrf1α and Nrf2 coordinately through direct and indirect mechanisms, so as to maintain normal redox homeostasis. Conversely, dysfunction of Nrf1α and Nrf2 (particularly its malfunction) leads to severe redox stress and cancer development possibly by the aberrant PTEN-PI3K-AKT signaling pathway.

It is inferable that almost abolishment of PTEN in malignantly growing Nrf1α−/− derived tumor cells results from an aberrant accumulation of Nrf2 protein, because rescue of PTEN expression occurs after Nrf2 is silenced, such that the existing Nrf1α−/−+siNrf2-derived tumor growth is dramatically repressed by knockdown of Nrf2. In turn, the aberrant accumulation of Nrf2 in Nrf1α−/− cells is caused by impaired PTEN expression. This is fully consistent with the pathology of PTEN−/− -leading cancer, in which the abnormal nuclear accumulation of Nrf2 is caused by impairment of GSK3β-directed β-TrCP-based proteasome-mediated degradation, as described by (Best et al., 2018; Rojo et al., 2014; Taguchi et al., 2014). In addition to impairment of the GSK3β-directed β-TrCP pathway, aberrant accumulation of Nrf2 is augmented by inhibition of Keap1-based proteasome-mediated degradation in Nrf1α−/− derived tumor cells. Noticeably, the Keap1 protein, rather than mRNA, levels are significantly reduced in Nrf1α−/− cells, albeit its binding partner p62, acting as a major regulator of Keap1 to the autophagic degradation (Kageyama et al., 2018), is strikingly down-regulated in Nrf1α−/− cells. Thus, we postulate that a p62-independent mechanism may account for the Keap1 protein degradation and also is reinforced in Nrf1α−/− cells. Yet, it cannot be ruled out that biosynthesis of Keap1 polypeptides may also be retarded during these conditions.

Several lines of evidence presented here demonstrate that Nrf2 is predominantly negatively regulated by Nrf1α because Nrf1α−/− enables Nrf2 to be released from the confinements by the PTEN-GSK3β-directed β-TrCP-based and
Keap1-based proteasomal pathways (Figure 5K). Consequently, accumulation of Nrf2 leads to aberrant activation of ARE-driven cytoprotective genes (e.g. HO-1, GCLM, NQO1) to shelter or promote Nrf1α+/−-driven tumor cells. In fact, These ARE-battery genes can be directly activated by Nrf1α, but some of these downstream genes could also be inhibited through braking control of the Nrf2 activity. Overall, distinct levels of Nrf1 alone or in cooperation with Nrf2 finely tune and also quantitatively regulate expression of diverse downstream genes to meet different cellular needs (Figures 4L, 5K). Thus, these resulting collective effects determine distinct phenotypes of animal xenograft tumor models as deciphered in this study (Figure 7H). Consistently, the malignant growth of Nrf1α+/−-derived tumor is substantially suppressed by knockdown of Nrf2, by comparison with Nrf1α+/−+siNrf2-derived tumor. Conversely, almost no solid tumor is formed in nude mice that have been inoculated by injecting inactive Nrf2−/−-atac-derived cells, albeit Nrf1 is slightly decreased with loss of Nrf2’s function. These demonstrate that Nrf1α acts as a dominant tumor suppressor principally by confining the oncogenicity of Nrf2. In turn, Nrf2 exerts a dominant tumor-promoting role in tumorigenesis and malignant growth, but it can also directly mediate the Nrf1 gene transcription to form a feedback regulatory loop. This is validated by further evidence revealing that, upon the presence of Nrf1 in canNrf22α-α-derived tumor cells, its growth is almost unaffected by constitutive activation of Nrf2, as well as antioxidant and detoxifying genes, when compared with wild-type Nrf1/2+/−-bearing tumor.

In an attempt to clarify those seemingly contradictory results obtained from loss of Nrf1α and its functional gain (i.e. ectopic over-expression), we have surprisingly found that there exists a mutual regulatory relationship between Nrf1α and Nrf2, thereby enabling both factors to elicit opposing and unifying roles in regulating distinct downstream genes (particularly ARE-driven cognate genes). Importantly, we have also discovered that that forced expression of Nrf1 enables the Nrf2 protein to be reduced, whereas loss of Nrf1α led to a significant increase in Nrf2 protein, but not its mRNA levels (Figure 4L). By contrast, both mRNA and protein levels of Nrf1 are increased by over-expression of Nrf2 or its constitutive activation, but also repressed by inactivation of Nrf2. Further experiments have unraveled no activation of the human Nrf2 promoter-driven Pnrf2-Luc reporter by Nrf1 or Nrf2, albeit mouse Nrf2 contains ARE sites as described (Kwak et al., 2002). However, the human Nrf1 promoter-driven Pnrf1-luc reporter is trans-activated by Nrf1 (at the locus Site-2) and Nrf2 (at the locus Site-1) (Figures 5, S6A). These findings demonstrate there are two (i.e. transcript and protein abundance) levels at which Nrf1α and Nrf2 have cross-talks with each other to influence the expression of ARE-driven genes. Thereby, synergistic or antagonistic effects of Nrf1α and Nrf2 depend on mutual competition or somehow coordination with spatiotemporally binding to the same or different ARE enhancers within downstream genes. Overall, such inter-regulatory cross-talks between Nrf1α and Nrf2 could be a vitally important strategy for the precision regulation of downstream genes. This rationale provides a better explanation of those complicated physio-pathological functions with distinct disease phenotypes exhibited in different models (as reviewed by Menegon et al., 2016; Zhang and Xiang, 2016).

Importantly, a hot controversy surrounds the roles of Nrf2 in the pro- or anti-cancer contexts, termed ‘the Nrf2 paradox’ (Menegon et al., 2016; Rojo de la Vega et al., 2018). This study has defined that function of Nrf2 is dictated by activation or inactivation of Nrf1α. This is because deterioration of Nrf1α+/−-tumor results from hyper-active Nrf2, along with decreased PTEN and activation of AKT signaling, but Nrf1/2−/−-tumor growth is unaffected by constitutive activation of Nrf2 when compared with caNrf22α-α-tumor. Consistently, it has been recently showed that Nrf2 acts as a tumor-promoting player, depending upon aberrant activation of the PI3K-AKT signaling pathway, whereas it serves as a tumor-preventing player through activating ARE-driven cytoprotective genes under normal activation conditions (Best et al., 2018). However, a similar subset of ARE-driven genes is also highly expressed in Nrf1α−/− and caNrf22α-α cell lines. Our findings demonstrate that the tumor-promoting role of Nrf2 is determined by loss of Nrf1α function, independent of those cytoprotective gene expressions. Even as a braking control of Nrf2 activity, Nrf1α may play a role for ‘decision-maker’ or ‘executor’ in the cell senescence and cancer progression, since a secretory phenotype of
senescent cells occurs by a Nrf2-independent mechanism (Wang et al., 2017), albeit the relevance to Nrf1 needs to be verified.

In conclusion, this study provides a panoramic view of mutual inter-regulatory cross-talks existing between Nrf1 and Nrf2 to determine quantitative expression of distinct downstream genes involved in different patho-physiological processes. Significantly, the axiomatic rationale underlying distinct animal xenograft tumor phenotypes has been also unraveled by transcriptome analysis of the genome-wide gene expression in Nrf1α−/−, Nrf1α−/−+siNrf2, Nrf2−/−ΔTA and caNrf2ΔO cell lines, by comparison with wild-type Nrf1/2+. Notably, an overwhelming majority of the PTEN-directed PI3K-AKT signaling cascades are strikingly activated in Nrf1α−/−, but rather repressed in Nrf2−/−ΔTA cells. Silencing Nrf2 leads to opposing expression of 87 genes in between Nrf1α−/− and Nrf1α−/−+siNrf2 cell lines. Although most cognate genes are, to different extents, co-regulated by Nrf1α and Nrf2, this study has highlighted about 30 of Nrf1α-specific downstream genes, and 38 of Nrf2-specific downstream genes. Among Nrf1α-regulated genes, those encoding A2M, EPHA8, FBXO2, KCND1, SLC2A3, SORL1, OLIG2, and RAPGEF4 may be responsible for the nervous system, albeit it is unclear whether they are relevant to those phenotypes of Nrf1α−/−-leading neurodegenerative diseases as reported by (Kobayashi et al., 2011; Lee et al., 2011). Only ACS52, FA2H, and KLF15 genes are associated with lipid metabolism, but it is required to determine their roles in relevant phenotypes, as described by (Bartelt et al., 2018; Hirotsu et al., 2014; Hou et al., 2018; Xu et al., 2005). By contrast, a portion of Nrf2-specific genes are critical for the development of various tissues and organs, neurons and cardiomyocytes, but none of the specific physio-pathological phenotypes in the Nrf2−/−ΔDBD mice are observed, implying their functions can be compensated. As such, the other Nrf2-specific genes may be involved in the development, movement and adhesion of epithelial cells, but it is unknown whether these gene functions enable Nrf2 to be endowed with its potent tumor-promoting roles in cancer progression and metastasis. All together, the malfunction of Nrf2, as a tumor promoter, is predominantly suppressed by Nrf1α that acts as a dominant tumor repressor. This pathophysiological process is tightly governed by endogenous regulatory networks. On the inside, there exist mutual opposing and unifying cross-talks between Nrf1α and Nrf2 at distinct levels. Concordantly, Nrf2 directly mediates transcription of the Nrf1 gene to form a coupled positive and negative feedback circuit, in order to monitor Nrf1 and Nrf2 functioning towards precision expression of distinct downstream genes.

**STAR METHODS**

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- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
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  - mouse embryonic fibroblasts (MEFs)
  - Human liver cell lines with distinct genotypes
- **METHODS DETAILS**
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  - Expression constructs and other oligos used for siRNA and MirRNA
  - Subcutaneous tumor xenografts in nude mice
  - Histology
  - Lipid staining
  - Cellular ROS staining
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  - Real-time quantitative PCR
  - Western blotting
  - Flow cytometry analysis of cell cycle and apoptosis

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The genome-wide transcriptomic analysis
QUANTIFICATION AND STATISTICAL ANALYSIS
Study approval

SUPPLEMENTAL INFORMATION
Supplemental Information includes ten figures and one table.

AUTHOR CONTRIBUTIONS
L.Q. designed and performed most of the experiments except indicated elsewhere, made all figures and wrote the manuscript draft. M.W. performed the statistical analysis of transcriptome data. Y.R. participated in the preparation of gene knockout cell lines. X.R. and S.H. participated in animal experiments. S.W. provided critical suggestion and invaluable materials to improve the work. Y.Z. designed and supervised this study, interpreted all data, generated project resources, and wrote the manuscript. All authors reviewed and commented on the manuscript. Meanwhile, these authors declare no competing financial and other interests.

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**STAR+METHODS**

**KEY RESOURCES TABLE**

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Software and Algorithms

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Yiguo Zhang (yiguozhang@cqu.edu.cn)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal care and use

In this study, the relevant animal experiments were indeed conducted according to the valid ethical regulations that have been approved. All mice were maintained under standard animal housing conditions with a 12-h dark cycle and allowed access ad libitum to sterilized water and diet. All relevant studies were carried out on 8-week-old male mice (with the license No. PIL60/13167) in accordance with the United Kingdom Animal (Scientific Procedures) Act (1986) and the guidelines of the Animal Care and Use Committees of Chongqing University and the Third Military Medical University, both of which were subjected to the local ethical review (in China). All relevant experimental protocols were approved by the University Laboratory Animal Welfare and Ethics Committee (with two institutional licenses SCXX-PLA-20120011 and SYXX-PLA-20120031).

Mouse embryonic fibroblasts (MEFs)
The wild-type MEF and MEF$_{\text{Nrf1}^{-/-}\triangle DBD}$ cell lines (in which $\triangle DBD$ represents deletion of mouse Nrf1 DNA-binding domain (DBD)-adjoining codon sequence) were provided as a gift by Dr. Akira Kobayashi (Doshisha University, Japan). Additional wild-type MEF and MEF$_{\text{Nrf2}^{-/-}\triangle DBD}$, and MEF$_{\text{Keap1}^{-/-}}$ were from Prof. John D. Hayes (University of Dundee, UK). In fact, it should also be credited to Prof. Masayuki Yamamoto (Tohoku University, Japan) because all relevant mouse lines were originally made in his laboratory.

**Human liver cell lines with distinct genotypes**

All four cell lines Nrf1$\alpha^{-/-}$, Nrf1$\alpha^{-/-}+$siNrf2, Nrf2$^{-/-}^{-\text{sta}}$ and caNrf2$^{2N}$ were created in this study. Their progenitor cells are the human hepatocellular carcinoma (HepG2) and/or another non-cancerous human liver (HL7702) cell lines. The latter two lines HepG2 and HL7702 are wild-type (Nrf1$^{+/+}$, Keap1$^{+/+}$) cells, because not any mutants in the Nrf1, Nrf2 and Keap1 genes are therein confirmed by sequencing. However, it is important to be noted that Nrf1 and its long TCF11 isoform are co-expressed at a ratio of 1:1 in HL7702 cells. By contrast, significantly decreased expression of Nrf1 is observed in HepG2 cells (Ren et al., 2016), while almost none of its longer TCF11 transcripts were detected. For relevant identification of these cell lines, see Figures 1 and S1.

**METHODS DETAILS**

**Cell culture and transfection**

Cells were grown in DMEM supplemented with 5 mM glutamine, 10% (v/v) foetal bovine serum (FBS), 100 units/ml of either of penicillin and streptomycin, in the 37 °C incubator with 5% CO$_2$. The experimental cells were transfected with indicated plasmids alone or in combination for 8 h using Lipofectamine® 3000 Transfection Kit (Invitrogen, USA), and then allowed for recovery from transfection in the fresh medium for 24 h before being subjected to indicated experiments.

**Expression constructs and other oligos used for siRNA and miRNA**

Expression constructs for human Nrf1, Nrf2, JUN and FOS were made by cloning each of their full-length cDNA sequences into a pcDNA3 vector, respectively. Notably, the other plasmids specifically for the genome-editing of Nrf1 or Nrf2 by Talens or CRISPR/Cas9 were created (in Figure S1) and identified (in Figure 1). Furtherly, we were made four specific luciferase reporters, which were driven by distinct gene promoter regions from the human Nrf1, Nrf2, COX1 and COX2. Different lengths of these gene promoter regions were amplified by PCR from their genomic loci and inserted into the PGL3-basic vector. In addition to these intact reporter genes $P_{\text{Nrf1-Luc}}$, $P_{\text{Nrf2-Luc}}$, $P_{\text{COX1-Luc}}$, $P_{\text{COX2-Luc}}$ and $miR22-ARE-Luc$, all these relevant ARE-specific mutant reporters were engineered. Moreover, double fluorescent reporters (i.e. PTEN-miR22b and COX1-miR22b) were created by cloning the 3’ UTR region sequences of COX1 and PTEN, that were amplified from reverse transcription PCR products and ligated into the psiCHECK2 vector. All primers and other oligos used for siRNAs and Mir-RNAs were synthesized by Sangon Biotech (Shanghai, China). The fidelity of all constructs used in this study was confirmed to be true by sequencing.

**Subcutaneous tumor xenografts in nude mice**

Mouse xenograft models were made by subcutaneous heterotransplantation of the human hepatoma HepG2 (i.e. Nrf1/2$^{+/+}$ or each derivate of Nrf1$^{1a^{-/-}}$, Nrf1a$^{-/-}+$siNrf2, Nrf2$^{-/-}^{-\text{sta}}$ and caNrf2$^{2N}$ cell lines into nude mice, as described (Morton and Houghton, 2007). Experimental human hepatoma cells (1 x $10^7$, allowed for growth in the exponential phase) were suspended in 0.2 ml of serum-free DMEM and were inoculated subcutaneously into the right upper back region of male nude mice (BALB/C$^\text{nu/nu}$, 6 weeks, 18 g, from HFK Bioscience, Beijing) at a single site. The procedure of injection into all mice was completed within 30 min, and the formation of the subcutaneous tumour xenografts was observed. Once the tumor xenografts emerged, their sizes were successively measured once every two days, until the
32nd day when the mice were sacrificed before the transplanted tumors were excised. The sizes of growing tumors were calculated by a standard formula (i.e. $V = \frac{ab^2}{2}$) and then are shown graphically ($n = 6$ per group). Subsequently, the tumor tissues were also subjected to the histopathological examination by the routine hematoxylin-eosin staining. Notably, all the relevant animal experiments in this study were indeed conducted according to the valid ethical regulations that have been approved.

**Histology**

Xenograft tumor tissues were immersed in 4% paraformaldehyde for overnight and then transferred to 70% ethanol. Individual tumor tissues were placed in processing cassettes, dehydrated through a serial of alcohol gradient, and embedded in paraffin wax blocks. Before staining, the tissue sections were de-waxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in PBS. Lastly, they were stained with hematoxylin and eosin (H&E), and visualized by microscopy.

**Lipid staining**

Experimental cells were seeded in 6-well plates and cultured with 200 μM sodium oleate (Solarbio, Beijing, China) medium. The cells were fixed for 30 min with 4% paraformaldehyde (AR1068, Boster Biological Technology, Wuhan, China) and then stained for 30 min with a solution of 3 g/L oil red O (A600395, Sangon Biotech, Shanghai, China). The stained cells were rinsed 3 times with 60 % of isopropyl alcohol (Kelong, Chengdu, China) before the red lipid droplets were visualized by microscopy.

**Cellular ROS staining**

Experimental cells were allowed for growth to an appropriate confluence in 6-well plates and then incubated in serum-free medium containing 10 μM of 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) (S0033, Beyotime, Shanghai, China) at 37°C for 20 min. Thereafter, the cells were washed three times with serum-free medium, before the green fluorescent images were achieved by microscopy.

**Luciferase reporter assay**

Equal numbers (1.0×10^5) of experimental cells were seeded into each well of the 12-well plates. After reaching 80% confluence, the cells were transfected with a Lipofectamine 3000 mixture with luciferase plasmids with or without other expression plasmids. In the pGL3 plasmid system, the Renilla-luciferase, which expression by pRL-TK plasmid, as an internal control for transfection efficiency. And in the psi-CHECK2 plasmid system, the Pyralis-luciferase activity is the internal control, while the Renilla-luciferase activity is the experimental test object. The luciferase activity was measured by using the dual-luciferase reporter assay system (E1910, Promega). The resultant data were normalized as a fold change (mean ± S.D) relative to the activity of the control group (at a given value of 1.0). The data presented here represent at least 3 independent experiments undertaken on separate occasions that were each performed in triplicate. Significant differences in the transcriptional activity were subjected to statistical analysis.

**Real-time quantitative PCR**

Experimental cells were subjected to isolation of total RNAs by using the RNAsimple Kit (Tiangen Biotech CO., Beijing). Subsequently, 500 ng of total RNAs were added in a reverse-transcriptase reaction to generate the first strand of cDNA (with Revert Aid First Strand Synthesis Kit from Thermo). The synthesized cDNA was served as the template for qPCR, in the GoTaq® qPCR Master Mix (from Promega), before being deactivated at 95°C for 10 min, and amplified by 40 reaction cycles of the annealing at 95°C for 15 s and then extending at 60°C for 30 s. The final melting curve was
validated to examine the amplification quality, whereas the mRNA expression level of β-actin served as an optimal internal standard control.

**Western blotting**

Experimental cells were harvested in a lysis buffer (0.5% SDS, 0.04 mol/L DTT, pH 7.5), which was supplemented with the protease inhibitor cOmplete Tablets EASYpack or phosphatase inhibitor PhosSTOP EASYpack (either 1 tablet per 10 mL, Roche, Germany). The lysates were denatured immediately at 100°C for 10 min, sonicated sufficiently, and diluted in 3× loading buffer (187.5 mmol/L Tris-HCl, pH 6.8, 6% SDS, 30% Glycerol, 150 mmol/L DTT, 0.3% Bromphenol Blue) at 100°C for 5 min. Subsequently, equal amounts of protein extracts were subjected to separation of proteins by SDS-PAGE containing 4-15% polyacrylamide, and then visualization by Western blotting with distinct antibodies as indicated. On some occasions, the blotted membranes were stripped for 30 min and then re-probed with additional primary antibodies. β-actin served as an internal control to verify equal loading of proteins in each of electrophoretic wells.

**Flow cytometry analysis of cell cycle and apoptosis**

Experimental cells (5 × 10⁵) were allowed for growth in 60-mm cell culture plate for 48 h and synchronization by 12-h starvation in a serum-free medium, before being treated with 10 μmol/L BrdU for 12 h. The cells were fixed for 15 min with 100 μl of BD Cytofix/Cytoperm buffer (containing a mixture of the fixative paraformaldehyde and the detergent saponin) at room temperature and permeabilized for 10 min with 100 μl of BD Cytoperm permeabilization buffer plus (containing fetal bovine serum as a staining enhancer) on ice. Thereafter, the cells were re-fixed and treated with 100 μl of DNase (at a dose of 300 μg/ml in PBS) for 1 h at 37 °C, in order to expose the incorporated BrdU, followed by staining with FITC conjugated anti-BrdU antibody for 60 min at room temperature. Subsequently, the cells were suspended in 20 μl of 7-amino-actinomycin D solution 20 min for the DNA staining and re-suspended in 0.5 ml of a staining buffer (i.e. 1 × DPBS containing 0.09% sodium azide and 3% heat-inactivated FBS), prior to flow cytometry analysis. Furthermore, additional fractions of cells (5 × 10⁵) were allowed for 48 h growth in 60-mm cell culture plate before being used for apoptosis analysis. The cells were pelleted by centrifuging at 1000 × g for 5 min and washed by PBS for three times, before being incubated for 15 min with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI) in 195 μl of the binding buffer, prior to flow cytometry analysis of cell apoptosis. The results being analyzed by the FlowJo 7.6.1 software.

**The genome-wide transcriptomic analysis**

Total RNAs were subjected to the sequencing by the Beijing Genomics Institute (BGI, www.genomics.org.cn) on the platform of BGISEQ-500 (contract No. F17FTSCCWJ1161). After removing the ‘dirty’ raw reads with data filtering, the clean reads were generated and mapped to the reference by using both HISAT (Kim et al., 2015) and Bowtie2 (Langmead et al., 2009) tools. Of note, gene expression levels were calculated by using the FPKM (Fragments Per Kilobase of exon model per Million mapped fragments) method with RSEM (Li and Dewey, 2011). Then, differentially expressed genes (DEGs) were identified with the criteria Fold-change ≥2 and diverge probability ≥0.8 by using the NOISeq (Tarazona et al., 2011). For the functional annotation, all DEGs were mapped to gene ontology (GO) terms in the database (http://www.geneontology.org/) and the pathway enrichment analysis of DEGs was performed by using KEGG (Kanehisa et al., 2008).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Significant differences were statistically determined using the Student’s t-test and Multiple Analysis of Variations (MANOVA). The data are here shown as a fold change (mean ± S.D.), each of which represents at least 3 independent
experiments that were each performed in triplicate.

**Study approval**

All experimental procedures were approved by the Animal Care and Use Committees of Chongqing University and the Third Military Medical University, both of which were subjected to the local ethical review (in China). Randomly assigned nude mice were injected subcutaneously with distinct cell lines as experimented.

**REFERENCES**


Ren, Y., Qiu, L., Lü, F., Ru, X., Li, S., Xiang, Y., Yu, S., and Zhang, Y. (2016). TALENs-directed knockout of the full-length transcription factor Nrf1a that represses malignant behaviour of human hepatocellular carcinoma (HepG2) cells Scientific Reports 7, Accepted for publication.


Figure S1. The human Nrf1- and Nrf2-specific gene-editing constructs

(A) Nrf1α-specific targeting constructs for TALEN-mediated gene editing. Both its left- and right- arms were designed for deletion of the first translation initiation codons of the Nrf1 gene (i.e. Nrf1α−/−).

(B) Nrf2-specific constructs for CRISPR/CAS9-directed gene editing. They were designed for deleting a fragment of the Nrf2 gene encoding most of both Neh4 and Neh5 domains (to yield an inactive Nrf2−/−ΔTA).

(C) Another Nrf2-specific editing construct by CRISPR/CAS9. It was designed for the dominant-active mutant of Nrf2.
so as to delete the sequence encoding the N-terminal Keap1-binding domain. The resulting mutant (i.e. caNrf2ΔN) was aligned with wild-type nucleotide sequence of Nrf2.

Figure S2

(A) Nrf1/2+/+, Nrf1α−/− and Nrf2−/−ΔTA cells were transfected with the Pcox2-luc and pRL-TK reporters for 12 h, and then treated with 100 nM of TPA for indicated lengths of time, before being measured for the luciferase activity. The data are shown as mean ± SEM (n = 3×3; $, p < 0.01 compared with the untreated control values).

(B) The above data are shown graphically.

Figure S3

(A) Schematic representation of potential upstream signaling to regulate COX2.

(B) Alterations in the indicated gene expression in Nrf1α−/−, compared with Nrf1/2+/+, cells. The data were obtained...
from transcriptome and are shown as mean ± SEM (n=3; *p<0.01; **p<0.001; $p<0.01; $$p<0.001).

(C to E) Nrf1α−/− cells were treated for 24 h with (C) 20 μM of JSH23, 25μM of CAP, (D) 10 μM of H-89, 1 μM of BAPTA-AM, or (E) 20 μM of SP600125, before COX2 was examined by Western blotting.

Figure S4

Figure S4. Activation of some AP-1 components in Nrf1α−/− cells
(A) The cartoon shows possible JNK signaling to downstream targets.
(B) The transcriptome analysis of major downstream genes regulated by JNK signaling. The data are shown as mean ± SEM (n=3, *p<0.01; $$p<0.001 compared with wild-type values)
(C) Either Pcox2-luc or PtRE-luc together with pRL-TK were co-transfected with each of indicated expression constructs or empty pcDNA3 vector and allowed for 24-h recovery, before being determined. The data are shown as mean ± SEM (n = 3×3; $, p<0.01; $$, p<0.001).
(D) The real-time qPCR analysis of distinct AP-1 subunits at their mRNA levels in Nrf1/2+/+, Nrf1α−/− and Nrf2−/−TA cells.
The data are shown as mean ± SEM (n= 3×3, *p<0.01; $, p<0.01; $$, p<0.001).
(E) Western blotting of JUN, FOS, and Fra1 abundances in Nrf1α−/− and Nrf1/2+/+ cells.
(F) Abundances of JUN, FOS, and Fra1 was visualized Western blotting of Nrf2−/−TA and Nrf1/2+/+ cells.
(G) Nrf1α−/− cells were treated with 4 μM of SR11302 for 24 h before COX2 were examined by Western blotting.
(H) Nrf1α−/− cells were allowed for knockdown by siJUN (60 nM) and siFOSL1 (60 nM) for 24 h, respectively, before COX2, Fra1 and JUN were determined by Western blotting.

(I) Nrf1/2+/− cells were subjected to silencing of siNrf2 (60 nM) and allowed for 24-h recovery, before Nrf2, HO1 and GCLM were visualized by immunoblotting.

![Figure S5](image)

**Figure S5.** Cross-talks between Nrf1 and Nrf2 to regulate COX2

(A) Identification of HL7702Nrf1α−/− by the genomic site-specific sequencing. The resulting mutant of Nrf1α was aligned with the wild-type nucleotide sequence.
(B) Distinctions of Nrf1, Nrf2 and COX2 in between HL7702\textsuperscript{Nrf1α−/−} and HL7702\textsuperscript{Nrf1α+/+} cells was observed by Western blotting.

(C) Subtle nuances in the abundances of Nrf1, Nrf2, COX2 and HO-1 in between MEF\textsuperscript{Nrf1+/+}, MEF\textsuperscript{Nrf1−/−}, MEF\textsuperscript{Nrf2+/+} and MEF\textsuperscript{Nrf2−/−} were determined by Western blotting.

(D) Alterations in the expression of Keap1, Nrf1, Nrf2, COX2 and HO-1 in between MEF\textsuperscript{Keap1+/+} and MEF\textsuperscript{Keap1−/−} were detected by Western blotting.

(E) Differences of Keap1, Nrf1, Nrf2, COX2, HO-1 abundances in between HepG2\textsuperscript{Keap1+/+} and HepG2\textsuperscript{Keap1−/−} were visualized by Western blotting.

(F) Differential expression of Nrf2, COX1, COX2, HO-1, GCLM and xCT at mRNA levels in Nrf1α−/− and Nrf1α+/+siNrf2 cells were determined by the transcriptome. The data are shown as mean ± SEM (n=3, *p < 0.01; $p < 0.01).

(G) Both Nrf2\textsuperscript{−/−ΔTA} and caNrf2\textsuperscript{ΔN} cell lines differentially expressed mRNA levels of Nrf1, Nrf2, COX1, COX2, xCT and Lpin1. The transcriptome FPKM data are shown as mean ± SEM (n=3, *p < 0.01; $p < 0.01).

**Figure S6**

(A) The COX1\textsuperscript{miR22b} was constructed as above, which contains miR-22 binding site which in the COX1’s 3’UTR.
region (upper). Nrf1/2−/− cells were co-transfected with COX1-miR22b or COX1-miR22b-mut, together with miR-22 or NC plasmids (A1), or pcDNA3, an expression construct for Nrf1 or Nrf2 (A2), and then allowed for 24-h recovery before being determined. The data are shown as mean ± SEM (n = 3×3, NS= no statistical difference).

(B) Nrf1/2−/− cells were co-transfected with the Pcox1-luc and pRL-TK (B1 to B3), plus pcDNA3 or indicated expression constructs for Nrf1, Nrf2 (B1), Jun, Fos or Jun+pFos (B3), and allowed for 24-h recovery, before being treated (B2), or were not treated (B1, B3), with 100 nM of TPA for 2-6 h, prior to being measured for the luciferase activity. The data are shown as mean ± SEM (n = 3×3, *p < 0.01, NS= no statistical difference).

Figure S7. Differences in transcriptional expression of proteasomal subunits regulated by Nrf1 and Nrf2

(A) Two cis-Nrf1/Nef2l1-regulatory locus sites (i.e. Site-1 and Site-2) exist in this gene promoter, as located (upper). The
nucleotide sequence of both Site-1 and Site-2 are shown.

(B) Immunoblotting with antibodies against ubiquitinated proteins (i.e. anti-ub) in Nrf1/2+/+ and Nrf1α−/− cells.

(C) Almost no or less anti-ub cross-reactivity with ubiquitinated proteins in Nrf1/2+/+ and Nrf2−ΔTA cells was observed.

(D) Significant decreases in the expression of most of the 26S proteasomal subunits and related proteins were detected in Nrf1α−/− cells when compared with those in Nrf1/2+/+. The transcriptome data are shown as mean ± SEM (n=3, *p<0.01; $ p<0.01).

(E) Almost no changes in transcriptional expression of most proteasomal and related genes in Nrf1/2+/+ and Nrf2−ΔTA cells. The transcriptome data are shown as mean ± SEM (n=3, *p<0.01; $ p<0.01).

Figure S8. Subtle nuances in distinct cell cycles and apoptosis processes

(A) Changes in expression of cell cycle-related genes in five distinct cell lines as indicated. The transcriptome data are...
shown as mean ± SEM (n=3, *p<0.01; $p<0.01).

(B to F) Flow cytometry analysis of apoptosis in five distinct cell lines as indicated. Abbreviations: UL, necrotic cells; UR, early apoptotic cells; LL, normal cells; LR, late apoptotic cells.

(G) The expression of *FTH1* and *FTL* genes were detected by transcriptome sequencing. The data are shown as mean ± SEM (n=3, *p<0.01; $p<0.01).

Figure S9. Opposite changes in DEGs measured from transcriptome in distinct cell lines

(A) Significant differences in the indicated DEGs responsible for PTEN-directed PI3K-AKT signaling pathways (also...
shown in Figure 7B & 7C) in between Nrf1α−/− and Nrf2−/−ΔTA cell lines are shown graphically, after normalization to relevant values measured from Nrf1/2+/+ cells by transcriptome sequencing (n=3).

(B to D) Opposite alterations in DEGs in between Nrf1α−/− and Nrf1α−/+siNrf2 cell lines after being normalized to those in Nrf1/2+/+ cells are shown in different ways. The major functions of these genes are also classified.

Figure S10. Opposite alterations in DEGs measured from transcriptome in Nrf2−/−ΔTA and caNrf2ΔN cells
These genes display opposite trends in their expression levels in between Nrf2−/−ΔTA and caNrf2ΔN, after normalization to relevant values measured from Nrf1/2+/+ cells by transcriptome sequencing (n=3). The major functions of these genes are also classified.
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<th>No.</th>
<th>Pathway</th>
<th>DEGs genes with pathway annotation (1000)</th>
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<th>Pvalue</th>
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<td>1</td>
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<td>ko04068</td>
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<td>145 (0.74%)</td>
<td>9.52E-05</td>
<td>ko04933</td>
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<td>Epithelial cell signaling in HeLa cells</td>
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Table S1. KEGG pathway enrichment analysis of DEGs