Loss of estrogen-related receptor α promotes hepatocarcinogenesis development via metabolic and inflammatory disturbances

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Estrogen-related receptor α (ERR α) is a key regulator of mitochondrial function and metabolism essential for energy-driven cellular processes in both normal and cancer cells. ERR α has also been shown to mediate bone-derived macrophage activation by proinflammatory cytokines. However, the role of ERR α in cancer in which inflammation acts as a tumor promoter has yet to be investigated. Herein we show that global loss of ERR α accelerates the development of diethylnitrosamine (DEN)-induced hepatocellular carcinoma. Biochemical and metabolomics studies revealed that loss of ERR α promotes hepatocyte necrosis over apoptosis in response to DEN due to a deficiency in energy production. We further show that increased hepatocyte death and associated compensatory proliferation observed in DEN-injured ERR α -null livers is concomitant with increased nuclear factor κ B (NF- κ B)-dependent transcriptional control of cytokine expression in Kupffer cells. In particular, we demonstrate that loss of ERR α -dependent regulation of the NF- κ B inhibitor I κ B α leads to enhanced NF- κ B activity and cytokine gene activation. Our work thus shows that global loss of ERR α activity promotes hepatocellular carcinoma by independent but synergistic mechanisms in hepatocytes and Kupffer cells, implying that pharmacological manipulation of ERR α activity may have a significant clinical impact on carcinogen-induced cancers.

nuclear receptor | liver cancer

Hepatocellular carcinoma (HCC) is a major cause of cancer deaths worldwide, particularly in countries with high risk factors that include greater exposure to aflatoxin B1, a fungal contaminant in dietary supplies, and to the hepatitis B and C viruses (1). HCC has also been linked with exposure to toxic chemicals such as polycyclic aromatic hydrocarbons and nitrosamines and is more frequent in individuals with cirrhosis associated with chronic inflammation (2, 3). HCC that closely resembles the human disease can be induced in mice with a single postnatal injection of the tumor initiator diethylnitrosamine (DEN) (4). In this model, DNA damage induced by the carcinogen promotes cell death, which leads to an inflammatory response by resident Kupffer cells that further stimulates tumor development fueled by compensatory proliferation of hepatocytes (5). It has been shown that nuclear factor κ B (NF- κ B) signaling, a critical intracellular pathway in the regulation of inflammation, plays a complex role in DEN-induced hepatocarcinogenesis, displaying antitumorigenic and procarcinogenic activity in hepatocytes and Kupffer cells, respectively. In hepatocytes, NF- κ B signaling protects against cell death, thus attenuating compensatory cell proliferation, while promoting the inflammatory response of Kupffer cells and production of hepatomitogens (5).

Estrogen-related receptor α (ERR α) is an orphan nuclear receptor that plays a central role in the control of energy metabolism (6). As a transcription factor, ERR α directly regulates the expression of genes required for mitochondrial biogenesis and function, including genes encoding enzymes and proteins participating in the tricarboxylic acid (TCA) cycle, pyruvate

metabolism, oxidative phosphorylation, and electron transport (7–11). Extensive phenotypic analyses of the ERR α -null mouse model have shown that ERR α specifies the energetic properties required for cell- and tissue-specific functions (12). In bone-derived macrophages, the expression of ERR α is stimulated by proinflammatory cytokines, and the presence of ERR α is required for efficient mitochondrial respiratory activities, production of reactive oxygen species (ROS), and pathogen clearance (13). In the liver, pharmacological or genetic inhibition of ERR α activity alters the expression profile of genes involved in the TCA cycle and lipid biogenesis, stimulates the accumulation of TCA cycle metabolites, and exacerbates hepatic hyperlipidemia induced by the anticancer drug rapamycin (11). ERR α also influences the proliferation of cancer cells by promoting a metabolic shift favoring the use of glucose as an energy source, a phenomenon referred to as the Warburg effect, and by regulating the expression of key oncogenes (14–17).

The link between ERR α as a regulator of energy metabolism, its role in inflammatory processes, and its role as a facilitator of cancer cell growth raised the question of whether ERR α also plays a role in inflammation-associated cancers. In this study, we examined the development of DEN-induced HCC in ERR α -null mice. Unexpectedly, our study reveals that loss of ERR α accelerates the progression of DEN-induced HCC despite reduced ROS levels and mitochondrial dysfunction in hepatocytes. Loss of ERR α impaired the DEN-stimulated energy-dependent apoptotic ability of hepatocytes but increased liver damage through

Significance

This study shows that absence of the orphan nuclear receptor estrogen-related receptor α (ERR α), a master regulator of cellular energy metabolism, predisposes mice to hepatocellular cancer development when exposed to a known carcinogen. Biochemical and metabolomics studies revealed that loss of ERR α promotes hepatocyte necrosis over apoptosis in response to a carcinogen due to a deficiency in energy production. In addition, we demonstrate that loss of ERR α -dependent regulation of the nuclear factor κ B (NF- κ B) inhibitor I κ B α leads to enhanced NF- κ B activity and cytokine gene activation. These findings have particular biological significance in view that rapamycin, a drug currently in use in various clinical settings, has been shown recently to decrease ERR α protein levels and activity in mouse liver.

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necrotic cell death. On the other hand, DEN treatment led to a greater activation of the NF- κ B pathway in the absence of ERR α in Kupffer cells, resulting in an increase in the inflammatory response and production of hepatomitogens, thereby augmenting compensatory proliferation of hepatocytes. Our work thus uncovers an important protective role for ERR α against inflammatory-related cancers and further highlights the complexity of ERR α action in the oncogenic process.

Results

Loss of ERR α Promotes DEN-Induced HCC. To investigate the role of ERR α in HCC development, 14-d-old wild-type (WT) and ERR α knockout (KO) male mice were injected with a single dose of the carcinogen DEN. Absence of ERR α significantly enhanced HCC development in response to DEN compared with WT mice (Table S1). Representative images of DEN-treated 9-mo-old livers show accelerated HCC development in ERR α -null mice characterized by an increase in the number of tumor nodules (Fig. 1A). At 9 mo of age, HCC was observed in all DEN-treated ERR α -null mice compared with only 25% in the DEN-treated WT mice (Fig. 1B and Table S1). At this time point, ERR α -null livers were found to have approximately five times more tumors corresponding to higher liver weights compared with WT in response to DEN (Fig. 1C and D). Moreover, DEN treatment resulted in greater liver injury in mice lacking ERR α relative to WT as demonstrated by a significantly higher level of serum alanine aminotransferase (ALT) in these mice (Fig. 1E). Histological examination of 9-mo-old livers of DEN-treated WT and ERR α -null mice is shown in Fig. 1F. Ki-67 labeling revealed significantly elevated levels of cell proliferation in the HCC region of livers lacking ERR α but not in the normal parenchyma (Fig. 1G). The increased tumor burden observed in DEN-treated ERR α KO mice also led to a significant decrease in survival of these mice compared with similarly treated WT mice (Fig. 1H).

DEN-Induced Hepatic Injury and Decreased Mitochondrial Function in ERR α -Null Hepatocytes. To further investigate the mechanisms underlying the protective action of ERR α in DEN-induced HCC before tumor formation, 6-wk-old WT and ERR α -null mice were subjected to a short-term exposure of a high dose of DEN. In agreement with the phenotype observed in the long-term DEN exposure experiment, mice lacking ERR α were found to exhibit significantly greater DEN-induced hepatic injury 48 h post-treatment as determined by increased serum ALT levels (Fig. 2A) and tumor protein p53 (p53) stabilization (Fig. 2B). The rise in p53 levels was accompanied by an increase in transcript levels of the p53-dependent proapoptotic genes *Bax* and *Pmaip1* (also known as *NOXA*) (Fig. 2C). We next explored whether ablation of ERR α affected mitochondrial function and ROS production. The expression of key ERR α nuclear-encoded mitochondrial target genes (*Mdh2*, *Sdhb*, *Cycs*, and *Cox5b*) were all significantly decreased in ERR α -null livers, a reduction that is independent of DEN treatment (Fig. 2D). As expected, DEN treatment led to a general increase in ROS abundance in WT livers (Fig. 2E). In contrast, the induction of ROS levels in response to DEN was blunted in livers lacking ERR α (Fig. 2E). This observation indicates that the greater DEN-driven liver injury observed in ERR α -null mice is not mediated by an increase in ROS-mediated tissue damage.

The reduction in ROS levels observed in ERR α -null livers indicates potential mitochondrial dysfunction. We next used cultured primary hepatocytes derived from WT and ERR α -null mice to further determine whether the loss of ERR α leads to altered metabolic energy production and mitochondrial biogenesis. Metabolomics analyses revealed significantly decreased phosphocreatine (PCr) levels associated with a more than twofold increase in the AMP/ATP ratio in ERR α -null hepatocytes compared with WT cells (Fig. 3A and B). Moreover,

Fig. 1. Loss of ERR α accelerates DEN-induced hepatic cancer. (A) Representative photographs showing HCC development in 9-mo-old livers of male WT and ERR α KO mice that were treated with DEN at 14 d old. Tumor nodules are indicated with arrowheads. (Scale bar, 1 cm.) (B) Tumor incidence, (C) number of tumors, (D) liver weight relative to whole body weight, and (E) circulating ALT levels in 9-mo-old DEN-treated WT and ERR α -null mice. Data represent means $\pm$ SEM. * $P < 0.05$. (F) Representative histological analyses of 9-mo-old DEN-treated WT and ERR α -null mice. Hematoxylin and eosin (H & E) staining and Ki-67 detection are shown. N, normal tissue; T, tumor. White arrowheads indicate proliferative cells. (Scale bar, 50 μ m.) (G) Percentage of Ki-67-positive cells in HCC (T) and normal parenchyma (N). Values represent means $\pm$ SEM. * $P < 0.05$. (H) Kaplan-Meier analysis shows decreased survival in DEN-treated mice lacking ERR α ($n = 11$). Log-rank test, $P = 0.0288$.

hepatocytes lacking ERR α have increased mitochondrial biogenesis characterized by a higher proportion of mitochondrial DNA versus genomic DNA compared with control cells (Fig. 3C). The data show that in the absence of ERR α , liver cells have decreased energy production due to impaired mitochondrial function, an observation that is validated by the increase in mitochondrial content to compensate for their inefficiency. The relationship between ERR α activity and cellular energy production was further investigated by treating WT and ERR α -null primary hepatocytes with tumor necrosis factor α (TNF α), an inflammatory cytokine known to induce inflammation-associated apoptotic cell death and ATP synthesis (18). TNF α treatment resulted in a robust increase in ATP production in WT hepatocytes associated with a decrease in the AMP/ATP ratio (Fig. 3

Fig. 2. Altered mitochondrial gene regulation in $ERR\alpha$ -null mice dampens acute DEN-induced ROS production. (A) Increased circulating ALT levels in mice lacking $ERR\alpha$ 48 h post-DEN injection in 6-wk-old mice. Values represent means $\pm$ SEM. $*P < 0.05$. (B) Western blot analysis and quantification of p53 in WT and $ERR\alpha$ -null liver extracts of acute DEN-treated mice. RPLP0 levels are shown as a loading control. Values represent means ($n = 4$) $\pm$ SEM. $*P < 0.05$. (C and D) Hepatic qRT-PCR expression analysis of p53 apoptotic target genes and metabolic $ERR\alpha$ target genes in livers of WT and $ERR\alpha$ -null mice following acute DEN treatment. Data are normalized to $RPLP0$ levels. Values represent means $\pm$ SEM. $*P < 0.05$. (E) ROS levels were measured in mitochondria from acute DEN-induced liver injured WT and $ERR\alpha$ -null mice. Values represent means $\pm$ SEM. $*P < 0.05$.

D and E). Strikingly, $TNF\alpha$ failed to induce energy production (Fig. 3D) or affect the AMP/ATP ratio (Fig. 3E) in $ERR\alpha$ -null primary hepatocytes.

Loss of $ERR\alpha$ Enhances Hepatocellular Necrosis. Increased hepatocyte cell death was observed in mice lacking $ERR\alpha$ in response to DEN as determined by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assays (Fig. S1 A and B). We then examined whether the increased cell death exhibited in DEN-injured livers of $ERR\alpha$ -null mice involved apoptosis. Although both genotypes were found to have similar hepatic protein levels of the intact caspases 3, 6, and 9, $ERR\alpha$ -null livers had significantly less cleaved caspase 3 levels following DEN injection (Fig. 4 A and B). The data suggest that the DEN-induced hepatic cell death observed in mice lacking $ERR\alpha$ occurs predominantly via necrosis rather than by apoptosis, an energy-consuming process (19). Indeed, $TNF\alpha$ induction of apoptotic cell death was only observed in WT hepatocytes as demonstrated by the presence of cleaved caspase 3 in these cells but not in $ERR\alpha$ -null hepatocytes (Fig. 4 C and D). The data suggest that $TNF\alpha$ -mediated cell death in $ERR\alpha$ -null hepatocytes likely occurs via necrosis, a non-energy-requiring process. To test this hypothesis, we next evaluated the influence of $ERR\alpha$ activity on the cellular release of the high-mobility group box 1 (HMGB1) protein. HMGB1 is specifically released by necrotic cells and used as a marker to distinguish between necrotic and apoptotic cell death (20). As WT and $ERR\alpha$ -null primary hepatocytes are cultured in media invariably contaminated by HMGB1 release from cells dying spontaneously, it is not reliable to measure $TNF\alpha$ -induced cell death in this system. To circumvent this problem, the release of HMGB1 was studied using the murine cell line Hepa 1–6 treated with or without compound 29 (C29), a highly specific $ERR\alpha$ inverse agonist (21), and/or $CoCl_2$, a chemical known to induce apoptosis (22). Treatment of cells with C29 alone resulted in loss of $ERR\alpha$ protein and release of HMGB1 in the media (Fig. 4E). Pharmacological inhibition of $ERR\alpha$ by C29 also suppressed $CoCl_2$ -mediated apoptotic cell death as demonstrated by the loss of caspase 3 cleavage and again promoted the release of HMGB1 in the media (Fig. 4E). Like WT Hepa 1–6 cells, cells overexpressing $ERR\alpha$ resulted in

$CoCl_2$ -dependent apoptotic cell death (Fig. 4F). Taken together, the data suggest that loss of $ERR\alpha$ decreases mitochondrial energy production, which results in a reduced ability of the cells to undergo ATP-dependent apoptosis. Thus, $ERR\alpha$ -null hepatocytes are more likely to die by necrosis upon receiving the chemical insult of DEN.

$ERR\alpha$ -Null Mice Exhibit Increased NF- κ B-Dependent Cytokine-Driven Compensatory Hepatocyte Proliferation in Response to DEN.

As loss of $ERR\alpha$ results in increased DEN-induced liver damage and hepatocellular death by necrosis, a phenomenon that would cause release of cellular constituents and activation of Kupffer cells, we next investigated a potential role for $ERR\alpha$ in cytokine-driven compensatory proliferation. First, the level of DNA synthesis as an indicator of cell proliferation was assessed by 5-bromo-2'-deoxyuridine (BrdU) labeling of livers acutely exposed to DEN. In agreement with the enhanced level of DEN-stimulated proliferation observed in 9-mo-old HCC-bearing mice (Fig. 1F), acute DEN treatment resulted in enhanced proliferation in $ERR\alpha$ -null livers 48 h posttreatment compared with DEN-treated WT mice (Fig. 5A). In response to acute DEN treatment, mice lacking $ERR\alpha$, compared with WT mice, were also found to have significantly elevated hepatic expression levels of the genes *Il1a*, *Il6*, *Tnf*, and *Hgf* encoding the proinflammatory cytokines interleukin (IL)-1 α , IL-6, TNF α , and hepatocyte growth factor (HGF), respectively (Fig. 5B). Note that HGF originates from stellate cells, whereas IL-1 α , IL-6, and TNF α are produced by Kupffer cells. Moreover, mice lacking $ERR\alpha$ were found to have increased serum levels of both IL-1 α and HGF in response to a 48-h DEN treatment (Fig. S2). Similarly, the endotoxin lipopolysaccharide (LPS) led to a greater induction in cytokine expression in isolated $ERR\alpha$ -null Kupffer cells compared with WT resident macrophages (Fig. 5C). Livers of DEN-treated $ERR\alpha$ -null mice were found to have higher nuclear levels of both NF- κ B subunits p50 and p65 (Fig. 5D). This observation suggests that

Fig. 3. Hepatocyte ATP production is $ERR\alpha$ -dependent. (A) Measurement of creatine, phosphocreatine, ATP, ADP, and AMP levels by LC-MS/MS from primary hepatocytes isolated from WT and $ERR\alpha$ -null mouse livers. (B) Relative abundance of AMP/ATP determined in A. Values represent means $\pm$ SEM. $*P < 0.05$. (C) Relative amounts of mitochondrial DNA/nuclear DNA between WT and $ERR\alpha$ -null primary hepatocytes. Values represent means $\pm$ SEM. $*P < 0.05$. (D) Measurement of ATP, ADP, and AMP levels by LC-MS/MS from cultured primary hepatocytes isolated from WT and $ERR\alpha$ -null mice treated with or without $TNF\alpha$ for 3 h. Values represent means $\pm$ SEM. $*P < 0.05$. (E) Relative abundance of AMP/ATP determined in D. Values represent means $\pm$ SEM. $*P < 0.05$.

Fig. 4. Loss of $ERR\alpha$ impairs cell stress-induced caspase-dependent apoptosis but promotes necrosis. (A and B) Western blot analysis and quantification of full-length and cleaved forms of caspase 3, 6, and 9 in WT and $ERR\alpha$ -null livers in response to acute DEN treatment. RPLP0 levels are shown as a loading control. Values represent means ($n = 4$) $\pm$ SEM. * $P < 0.05$. (C) Western blot analysis of $ERR\alpha$ and full-length and cleaved forms of caspase 3 and 9 in WT and $ERR\alpha$ -null primary hepatocytes treated with or without $TNF\alpha$ for 3 h. RPLP0 levels are shown as a loading control. (D) Immunofluorescence detection of cleaved caspase 3 (green) as a marker of apoptosis in $TNF\alpha$ -treated WT and $ERR\alpha$ -null primary hepatocytes. Cytoplasm is stained with α -Tubulin (red), and nuclei are stained with DAPI (blue). (E and F) Western blot analysis of $ERR\alpha$ and full-length and cleaved forms of caspase 3 in Hepa 1-6 cells treated with vehicle (control) or $CoCl_2$ with or without compound 29 (C29) or a vector overexpressing $ERR\alpha$. RPLP0 levels are shown as a loading control. HMGB1 protein was detected in the cultured media.

the increased DEN-induced cytokine-associated compensatory proliferation observed in $ERR\alpha$ -null livers may result in an intensification of NF- κ B activity in these cells. A recent ChIP-seq study identified NF- κ B binding events in the regulatory regions of these differentially regulated cytokines (23). We thus tested whether livers of DEN-treated mice lacking $ERR\alpha$ display increased recruitment of p65 to the NF- κ B response elements present in these cytokine genes. As shown in Fig. 5E and Fig. S34, binding of p65 to these sites was significantly augmented in DEN-treated $ERR\alpha$ -null livers. The data imply that $ERR\alpha$ may act as a repressor of NF- κ B signaling. Analysis of our recent mouse liver $ERR\alpha$ ChIP-seq study (11) revealed that the gene *Nfkb1a*, which encodes the NF- κ B suppressor $I\kappa$ B α , is a direct transcriptional target of $ERR\alpha$. $I\kappa$ B α acts as an inhibitor of NF- κ B signaling by sequestering NF- κ B dimers in the cytoplasm, thereby preventing NF- κ B-dependent transcriptional activity (24). $ERR\alpha$ ChIP experiments in both mouse liver and the macrophage cell line Raw 264.7 validate that $ERR\alpha$ is indeed recruited to the gene *Nfkb1a* at a regulatory region containing the consensus ERR response element, TCAAGGTCA (Fig. 5F and Fig. S3B). Decreased expression of *Nfkb1a* was found in both $ERR\alpha$ -null liver extracts and isolated primary Kupffer cells compared with control (Fig. S4A and B). In addition, treatment of Raw 264.7 cells with the $ERR\alpha$ inverse agonists C29 and XCT790 or a vector expressing $ERR\alpha$ resulted in decreased or increased $I\kappa$ B α transcript and protein levels, respectively (Fig. S4C–E and Fig. 5G–I). Moreover, livers of WT and $ERR\alpha$ -null mice subjected to an acute DEN treatment show reduced *Nfkb1a* mRNA and protein levels (Fig. 5J and K, respectively). Taken together, the data identify $ERR\alpha$ as a direct repressor of NF- κ B signaling.

Discussion

It is well-established that inflammatory-related diseases increase the risk of various types of cancer including liver, colon, gastric,

ovarian, and prostate cancer (25–29). Chronic inflammation is an important element of HCC development and progression and is associated with proliferation, angiogenesis, and metastasis (26). In addition, there is a clear connection between metabolic dysfunction and the immune response (30, 31). The generation and utilization of energy is a key process during inflammation, and given the known function of $ERR\alpha$ as a global regulator of energy metabolism (6) and its importance in pathogen resistance (13) and in various types of cancer (32), we sought to investigate the role of $ERR\alpha$ in inflammation-related HCC development.

Our work revealed that $ERR\alpha$ -null mice have an increased susceptibility to HCC initiation and progression. We show that loss of $ERR\alpha$ results in increased incidence and hepatic tumor number compared with WT mice following administration of DEN, a known carcinogen, resulting in premature lethality. Furthermore, DEN was found to induce greater liver damage assessed by circulating ALT levels and p53 function accompanied by increased hepatocellular death in $ERR\alpha$ -null mice. Although DEN has been shown to induce liver injury and hepatocyte cell death via ROS accumulation (33), we observed an inverse relationship between ALT and ROS levels in mice lacking $ERR\alpha$ in response to DEN. These results indicate that the liver damage in these mice is not predominantly mediated by intracellular ROS levels and that the lack of ROS may interfere with proper defense mechanisms against tumorigenesis such as apoptosis. Impaired ROS production in the absence of $ERR\alpha$ has also been previously observed in $ERR\alpha$ -null macrophages treated with the proinflammatory cytokine, $IFN-\gamma$, leading to a weakened response of bone-derived macrophages to pathogenic insults (13). However, in the context of HCC, loss of $ERR\alpha$ was found to enhance the inflammation-driven compensatory proliferation in response to increased DEN-stimulated cell death.

In vivo and in vitro studies provide evidence that the DEN-induced cell death in $ERR\alpha$ -null mice occurs via necrosis in contrast to ATP-dependent apoptosis. As previously observed, mice lacking $ERR\alpha$ were found to display decreased hepatic transcript levels of $ERR\alpha$ mitochondrial target genes (7, 9, 11). The mitochondrial dysfunction exhibited in $ERR\alpha$ -null mice was associated with decreased hepatic PCr energy reserves and increased AMP/ATP ratio. The results are in accordance with the decreased energy supply found in hearts of mice lacking $ERR\alpha$ (34). $TNF\alpha$ -induced cell death in WT primary hepatocytes enhanced ATP production required to undergo caspase-dependent apoptotic cell death, a process that was lost in $ERR\alpha$ -null hepatocytes. Our data show that in contrast to cells overexpressing $ERR\alpha$, cells lacking $ERR\alpha$ activity mediate chemically induced cell death primarily via necrosis, a non-energy-demanding process, rather than ATP-dependent apoptosis. These findings are particularly interesting in light of reports that tumor growth stimulated by necrotic death is mediated by compensatory proliferation (5) and that approaches to treat cancer by increasing apoptotic cell death and/or suppressing necrosis in tumors are studied (35).

Increased cell death observed in livers of DEN-injured $ERR\alpha$ -null mice was accompanied by enhanced compensatory proliferation. Activation of NF- κ B signaling is a hallmark of various inflammatory-related diseases and inflammation-associated cancers (36). NF- κ B signaling is activated by various stimuli including cytokines and necrotic effectors to mediate an inflammatory response. We provide evidence for a role of $ERR\alpha$ as a direct positive regulator of the NF- κ B inhibitor, $I\kappa$ B α , capable of sequestering NF- κ B in the cytoplasm. Inhibition or activation of $ERR\alpha$ in macrophage cells results in decreased or increased transcript and protein levels of $I\kappa$ B α . In vivo, we demonstrate that loss of $ERR\alpha$ results in enhanced hepatic NF- κ B nuclear translocation. Consequently, we observed increased transcript levels of NF- κ B proinflammatory cytokine targets including IL-1 α , IL-6, $TNF\alpha$, and HGF in livers of DEN-treated mice lacking

Fig. 5. Loss of *ERRα* stimulates an NF- κ B-dependent inflammatory response in DEN-injured liver. (A) Representative levels of BrdU labeling reflecting compensatory proliferation in DEN-injured WT and *ERRα*-null livers following a 48-h treatment are shown. The BrdU-positive cells (dark brown) are indicated with arrowheads. Quantification of BrdU-positive cells is shown. Values represent means ($n = 4$) $\pm$ SEM. $*P < 0.05$. (Scale bar, 100 μ m.) (B) Cytokine qRT-PCR expression analysis in livers of WT and *ERRα*-null mice following acute DEN treatment. Data are normalized to *RPLP0* levels. Values represent means $\pm$ SEM. $*P < 0.05$. (C) Cytokine qRT-PCR expression analysis in cultured primary Kupffer cells isolated from WT and *ERRα*-null mice following LPS treatment. Data are normalized to *RPLP0* levels. Values represent means $\pm$ SEM. $*P < 0.05$. (D) Western blot analysis of *ERRα* and the NF- κ B subunits p65 and p50 in liver cytoplasmic and nuclear extracts of WT and *ERRα*-null mice treated with or without DEN for 48 h. Lamin B1 and Tubulin levels are shown as a loading control for nuclear and cytoplasmic fractionation, respectively. (E) Standard ChIP qPCR analysis of p65 occupancy on NF- κ B target genes in livers of WT and *ERRα*-null mice treated with or without DEN for 48 h. Data are normalized to the enrichment of p65 at two control regions. Values represent means $\pm$ SEM. $*P < 0.05$. (F) Standard ChIP qPCR analysis of *ERRα* occupancy on *Nfkb1a* in mouse liver and the mouse macrophage cell line Raw 264.7. *Esrra* promoter, positive control. (G–I) Western blot analysis of *ERRα* and *IκBα* levels in Raw 264.7 cells treated with or without C29, XCT790, or a vector expressing *ERRα* for 48 h. *RPLP0* levels are shown as a loading control. (J) qRT-PCR expression analysis of the *Nfkb1a* in livers of WT and *ERRα*-null mice following acute DEN treatment. Data are normalized to *RPLP0* levels. Values represent means $\pm$ SEM. $*P < 0.05$. (K) Western blot analysis and quantification of *IκBα* in liver extracts of WT and *ERRα*-null mice exposed to an acute DEN treatment. *RPLP0* levels are shown as a loading control. Values represent means ($n = 4$) $\pm$ SEM. $*P < 0.05$.

ERRα. IL-1 α has been previously linked to necrotic cell death (33), and DEN-induced IL-6 production has been shown to lead to a liver regenerative response through hepatocyte proliferation (37). In addition, IL-6 has been found to prevent apoptosis by inducing Bcl-2 and Bcl2l1 (Bcl-xl) levels (38, 39). The genes encoding these two apoptotic regulators were found to be significantly up-regulated in livers of *ERRα*-null mice in response to acute DEN treatment (Fig. S5). In addition, previous studies have demonstrated that TNF α can mediate a hepatocyte damage response via JNK activation (40, 41). Moreover, the growth factor HGF secreted from stellate cells is known to promote DNA synthesis and hepatocyte proliferation during liver regeneration following injury (42). Furthermore, a greater induction in NF- κ B cytokine target gene expression was observed in LPS-treated Kupffer cells lacking *ERRα* compared with WT-treated cells. Our findings thus identify a previously unknown molecular crosstalk between *ERRα* and NF- κ B signaling.

The increased susceptibility to DEN-induced HCC initiation and progression in mice lacking *ERRα* is remarkable given that they exhibit impaired mitochondrial function. Results presented in our report indicate that the accelerated development of DEN-induced HCC in *ERRα*-null mice involves increased cytokine production as a consequence of hepatocyte necrotic death and compensatory proliferation due to the loss of *ERRα* repression of NF- κ B signaling in macrophages. These findings have particular biological significance in view that rapamycin, an mTOR inhibitor currently in use in various clinical settings, has been

shown recently to decrease *ERRα* protein levels and activity in mouse liver (11). In addition, molecules such as C29, a selective and bioavailable *ERRα* inhibitor, are being developed for the treatment of metabolic disorders including obesity and diabetes (21). This study thus indicates that positive modulation of *ERRα* activity could be envisioned to treat inflammatory-related diseases and cancer.

Methods

Animals. WT and *ERRα* KO mice (43) in a C57BL/6J background were housed in a pathogen-free facility at McGill University and fed standard chow with water provided ad libitum. All mouse experiments were approved and performed in accordance with the McGill Facility Animal Care Committee and the Canadian Council on Animal Care. For hepatocarcinogenesis experiments, 14-d-old mice were administered DEN (25 mg/kg body weight; N0258; Sigma) in a 0.9% saline solution by i.p. injection. The animals were monitored thereafter by palpation for tumor formation and killed by cervical dislocation at the indicated time points for serum and liver isolation. For acute DEN experiments, 6-wk-old mice were administered DEN (100 mg/kg body weight) in a 0.9% saline solution by i.p. injection, and livers were isolated at the indicated time points following injection. For the assessment of in vivo hepatocyte proliferation following acute DEN treatment, mice were injected with BrdU (1 mg per mouse; B5002; Sigma) 2 h before they were killed.

Cell Culture. All cell culture reagents were from Life Technologies. The Raw 264.7 murine macrophage cell line, Hepa 1–6 murine hepatoma cell line, and primary WT and *ERRα*-null hepatocytes and Kupffer cells were maintained at 37 $^{\circ}$ C in a 5% CO $_2$ atmosphere in DMEM supplemented with 10% (vol/vol) FBS, penicillin (100 U/mol), and streptomycin (100 μ g/mL). Raw 264.7 cells

were first plated in media containing 2% FBS, and the next day the cells were treated daily for 2 d with either vehicle (DMSO; C6164; Sigma), 5 μ M compound 29, or 1 μ M XCT790 (X4753; Sigma). For overexpression studies, Raw 264.7 cells (3×10^6) were plated in 6-cm plates with media containing 2% FBS, and the next day the cells were transfected with either 7 μ g of empty vector [cytomegalovirus-based expression plasmid (CMX)] or CMX-mERR α using FuGENE HD transfection reagent (FuGENE HD:DNA ratio = 3.5:1; E2311; Promega) and were harvested 48 h later. Hepa 1–6 cells were treated daily over a 3-d period with either vehicle (DMSO) or 10 μ M compound 29 in the presence or absence of 100 μ M cobalt chloride (CoCl₂; 202185; Sigma). For overexpression studies, Hepa 1–6 cells were transfected with either empty vector (CMX) or CMX-mERR α using HiPerFect reagent (Qiagen) and treated with or without 100 μ M CoCl₂ for 2 d. Primary hepatocytes isolated and cultured from WT and ERR α KO mice were treated with vehicle (PBS) or 10 ng/mL TNF α (410-TRNC-010; R&D Systems) for 3 h. Primary Kupffer cells isolated and cultured from WT and ERR α KO mice were treated for 3 h with either vehicle (PBS) or 10 ng/mL LPS (L3012; Sigma).

Statistical Analysis. Data are reported as mean $\pm$ SEM. Differences between means were obtained by Student *t* test, and the log-rank (Mantel–Cox) test was performed using GraphPad Software (GraphPad Inc.).

Isolation of primary hepatocytes and Kupffer cells, ALT and ROS measurements, Western blotting, histological and immunohistological analyses, ChIP, RNA isolation, qRT-PCR, mitochondrial and nuclear DNA quantification, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) detection of energy metabolites are described in *SI Methods*. Primers used for ERR α and p65 ChIP experiments as well as qRT-PCR analyses are shown in *Tables S2–S4*, respectively.

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