Cooperation Between the NRF2 Pathway and Oncogenic β-catenin During HCC Tumorigenesis

Mathilde Savall,1 Nadia Senni,1 Isabelle Lagoutte,1 Pierre Sohier,2 Renaud Dentin,1 Beatrice Romagnolo,1 Christine Perret1,1 and Pascale Bossard1

CTNNB1 (catenin beta 1)–mutated hepatocellular carcinomas (HCCs) account for a large proportion of human HCCs. They display high levels of respiratory chain activity. As metabolism and redox balance are closely linked, tumor cells must maintain their redox status during these metabolic alterations. We investigated the redox balance of these HCCs and the feasibility of targeting this balance as an avenue for targeted therapy. We assessed the expression of the nuclear erythroid 2 p45-related factor 2 (NRF2) detoxification pathway in an annotated human HCC data set and reported an enrichment of the NRF2 program in human HCCs with CTNNB1 mutations, largely independent of NFE2L2 (nuclear factor, erythroid 2 like 2) or KEAP1 (Kelch-like ECH-associated protein 1) mutations. We then used mice with hepatocyte-specific oncogenic β-catenin activation to evaluate the redox status associated with β-catenin activation in preneoplastic livers and tumors. We challenged them with various oxidative stressors and observed that the β-catenin pathway activation increased transcription of Nfe2l2, which protects β-catenin-activated hepatocytes from oxidative damage and supports tumor development. Moreover, outside of its effects on reactive oxygen species scavenging, we found out that Nrf2 itself contributes to the metabolic activity of β-catenin-activated cells. We then challenged β-catenin activated tumors pharmacologically to create a redox imbalance and found that pharmacological inactivation of Nrf2 was sufficient to considerably decrease the progression of β-catenin-dependent HCC development.

Conclusion: These results demonstrate cooperation between oncogenic β-catenin signaling and the NRF2 pathway in CTNNB1-mediated HCC tumorigenesis, and we provide evidence for the relevance of redox balance targeting as a therapeutic strategy in CTNNB1-mutated HCC. (Hepatology Communications 2021;5:1490-1506).

Tumorigenesis is a complex process involving multiple modifications promoting proliferation and preventing cell death. The maintenance of neoplastic cell proliferation and growth requires a metabolic rewiring to provide essential macromolecules and energy.1 Since the first observations that tumor cells displayed higher glucose consumption through a glycolytic pathway, it has been shown that there are numerous metabolic pathways, from enhanced glutaminolysis to increased fatty acid oxidation, that can be altered to favor tumor survival and progression.2-4

Although altering their metabolic activity, tumor cells also have to maintain a redox status compatible with survival, as metabolism and redox balance are closely linked—mostly through the production

Abbreviations: APAP, acetaminophen; Apc, adenomatous polyposis coli; ARE, antioxidant response element; ASP, acid-soluble products; ATP, adenosine triphosphate; CYP2E1, Cytochrome P450 Family 2 Subfamily E Member 1; DHE, dihydroethidium; ETC, electron transfer chain; FADH2, Flavin adenine dinucleotide; FAO, fatty acid oxidation; GCLC, Glutamate-Cysteine Ligase Catalytic Subunit; GPX, Glutathione Peroxidase; GSH, reduced glutathione; GSS, Glutathione Synthetase; GSSG, oxidized glutathione; GSTA, Glutathione S-Transferase Alpha; GSTM, Glutathione S-Transferase Mu; HCC, hepatocellular carcinoma; HMOX1, Heme Oxygenase 1; KEAP1, Kelch-like ECH-associated protein 1; MDA, malondialdehyde; mRNA, messenger RNA; NAC, N-acetyl cysteine; NFE2L2, Nuclear factor erythroid-derived 2-like 2; NRF2, nuclear erythroid 2 p45-related factor 2; NQO1, NAD(P)H:quinone oxidoreductase-1; PHGDH, Phosphoglycerate Dehydrogenase; PPARα, peroxisome proliferator-activated receptor alpha; PRDX, Peroxiredoxin 1; ROS, reactive oxygen species; siRNA, small interfering RNA; TCGA, The Cancer Genome Atlas; TXN, Thioredoxin; TXNRD, Thioredoxin Reductase; γH2AX, phosphorylated H2A histone family member X.

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of reactive oxygen species (ROS) by the oxidative phosphorylation pathway and through the production and the use of reductive entities such as nicotinamide adenine dinucleotide phosphate (NADPH) by different metabolic routes. Due to the intensification of their metabolism during cancer development and their presence in hypoxic environments, most malignant cells are in a pro-oxidative state.

One crucial regulator of cell defense against oxidative stress is the transcription factor nuclear erythroid 2 p45-related factor 2 (NRF2) encoded by nuclear factor erythroid 2 like 2 (NFE2L2), as it controls an enzymatic program involved in ROS detoxification, through the transcriptional regulation of components of the glutathione system, NADPH regeneration, heme metabolism, and phase I and phase II detoxification enzymes. Under basal conditions, NRF2 is maintained in the cytoplasm through its binding to the adaptor Kelch-like ECH-associated protein 1 (KEAP1)/Cul3-dependant ubiquitin ligase complex, leading to its degradation by the proteasome. Following oxidation of the KEAP1 cysteine residues, the NRF2/KEAP1 complex is dissociated, and NRF2 is stabilized and translocated to the nucleus. NRF2 stabilization is therefore highly dependent on the redox status of cells. However, constitutive activation of NRF2 by gain-of-function mutations in NFE2L2 or loss of function in KEAPI or other mechanisms affecting components of the NRF2 pathway have been observed in numerous cancers, indicating the need for transformed cells to constantly keep their ROS in check.

In response to physiological cues, ROS act as signaling molecules, affecting the activity of protein kinases and phosphatases controlling various pathways such as cell cycle, autophagy, or metabolism. Conversely, ROS can react with and modify all classes of biological molecules. An accumulation of ROS is therefore likely to have deleterious effects, altering cellular functions and triggering cell death. The survival and growth of cancer cells are therefore dependent on their ability to buffer excess oxidants. If the transformed cells manage to circumvent apoptosis, ROS-induced alterations can then contribute to tumorigenesis by increasing the instability of the nuclear genome and through signaling pathways affecting neoplastic cell proliferation. Given the role of redox signaling in cancer cell survival, effective treatment strategies targeting the oxidative vulnerability of these cells have been sought, either based on ROS scavenging to limit oxidative damage, or by disrupting the ROS/antioxidant defense (AOD) equilibrium to attain ROS levels beyond the threshold for damage to the transformed cells.

Hepatocellular carcinoma (HCC) is the fourth leading cause of death from cancer worldwide. HCC mostly occurs in a context of chronic liver inflammation associated with hepatitis, alcohol consumption, genotoxic exposure, or obesity. Based on genomic approaches, HCCs can be classified into two main subclasses: a proliferating subclass with high levels of chromosomal instability and containing...
HCC with mutations in TP53 or AXIN1. The second class includes tumors with activating mutations in CTNNB1, encoding β-catenin. HCCs with CTNNB1 mutations, referred to hereafter as CTNNB1 HCCs, display chromosomal stability, with a lower proliferative index and a less aggressive phenotype. We previously showed that CTNNB1 HCCs had a distinctive metabolism, making very efficient use of fatty acid oxidation (FAO) as their main source of energy and with enhanced mitochondrial respiratory chain activity. Here, following up on our observations of enhanced respiratory chain activity in β-catenin-activated preneoplastic livers, we characterized their redox balance. We found that the β-catenin pathway activation led to an increase in ROS production properly balanced by an increase in Nrf2 activity. The NRF2 program in human CTNNB1 HCCs was activated in a manner largely independent of NFE2L2 or KEAP1 mutations. These results reveal cooperation between the oncogenic β-catenin and NRF2 pathways during CTNNB1-mediated HCC tumorigenesis. This work also provides evidence in support of targeting the redox balance as a pertinent therapeutic strategy in CTNNB1 HCCs.

Materials and Methods

ANIMAL PROCEDURES

Mice carrying two floxed alleles on the 14th exon of the adenomatous polyposis coli (Apc) gene (Apclox/lox mice) were interbred with TTR-CreTam mice to generate Apclox/lox/TTR-CreTam mice and control Apclox/lox mice. The mouse used to model both pretumoral and tumor development for CTNNB1-activated human tumors has been described before: 2-month-old to 4-month-old male Apclox/lox/TTR-CreTam mice receiving intraperitoneal injections of 1.5 mg tamoxifen (MP Biomedicals, Santa Ana, CA) constituted the pretumoral model. This activation led to the panlobular expression of the surrogate marker for β-catenin activation, glutamine synthetase (Glul) (Supporting Fig. S1A). We assessed basal ROS accumulation in the hepatocytes of ApcHepKO and control mice (Fig. 1A). An analysis of superoxide ion (O2•−) accumulation on liver sections based on oxidized dihydroethidium (DHE) fluorescence staining revealed an absence of difference between ApcHepKO mice and control hepatocytes (Fig. 1A and Supporting Fig. S1B). A slightly significant lower H2O2 accumulation was observed in ApcHepKO hepatocytes than in control hepatocytes, suggesting that ApcHepKO have lower intracellular ROS (Fig. 1C). We challenged number of hepatocytes. These mice are referred to here as ApcTumLIV mice, and the HCCs developing in these mice are referred to as Apc HCCs. Tumor development was followed weekly, by 2D ultrasound. For in vivo N-acetyl cysteine (NAC) treatment, drinking water was supplemented with 12.5 mg/mL NAC, leading to a daily intake of 200 mg/kg, and the water was changed every 3 days for 3 weeks. Mice were housed under a 12-hour light/dark cycle in a controlled-temperature environment (21°C), and were fed ad libitum with a standard laboratory chow diet (SAFE 03) in specific pathogen-free conditions. All experiments were carried out during the light cycle.

All animal procedures were performed in accordance with French government regulations, with the approval of the Paris-Descartes Ethics Committee for animal experimentation, under protocol CEEA34. CP077.12. Animal experimentation permit number A-75-1845 was obtained from the French Ministère de L’enseignement Supérieur et de la Recherche.

Results

RESPONSE OF HEPATO CYTES DISPLAYING β-CATENIN ACTIVATION TO PRO-OXIDATIVE TREATMENTS

We investigated the redox status of β-catenin ApcHepKO mice, which we described in previous studies. Panlobular oncogenic β-catenin activation was achieved by acute Apc deletion in hepatocytes, in a pretumoral model, here named ApcHepKO. This activation led to the panlobular expression of the surrogate marker for β-catenin activation, glutamine synthetase (Glul) (Supporting Fig. S1A). We assessed basal ROS accumulation in the hepatocytes of ApcHepKO and control mice (Fig. 1A). An analysis of superoxide ion (O2•−) accumulation on liver sections based on oxidized dihydroethidium (DHE) fluorescence staining revealed an absence of difference between ApcHepKO hepatocytes and controls or primary hepatocytes in culture, confirmed by fluorescence quantification (Fig. 1A and Supporting Fig. S1B). A slightly significant lower H2O2 accumulation was observed in ApcHepKO hepatocytes than in control hepatocytes, suggesting that ApcHepKO have lower intracellular ROS (Fig. 1C). We challenged
the cells with hypoxia, known to increase ROS production through the electron transfer chain (ETC). Enhanced oxidized DHE staining was observed both in the livers of control mice maintained for 24 hours under 10% O₂ and in primary hepatocytes from control mice cultured for 45 minutes under 1%
O2, whereas this response was blunted in livers and primary hepatocytes from ApcHepKO mice (Fig. 1A). We challenged primary hepatocytes with rotenone (10 µM for 3 hours), which induces ROS production. This led to a sharp increase in both DHE staining and H2O2 production, but this increase was significantly smaller in ApcHepKO hepatocytes (Fig. 1B,C). Similar results were obtained with 5 mM paraquat, a potent ROS inducer (Fig. 1B,C). DHE fluorescence and H2O2 production were prevented by adding the antioxidant, NAC, to the culture medium (Fig. 1C and Supporting Fig. S1C). Supraphysiological ROS accumulation causes deleterious modifications to cell components, such as lipids, proteins, and DNA. We investigated lipid peroxidation by quantifying malondialdehyde (MDA), a naturally occurring product of lipid peroxidation by measuring MDA levels in ApcHepKO livers or primary hepatocytes with oncogenic β-catenin activation(20) hepatocytes with Keap1 expression unaffected (Fig. 2B,C and Supporting Fig. S1C). Nrf2 activity in β-catenin-activated hepatocytes was also confirmed by its trans-activating capacity of an ARE upstream from a luciferase reporter gene (ARE luciferase) in hepatocytes using a small interfering (siRNA) strategy targeting Nfe2l2 mRNA. Transfection with Nfe2l2 siRNA resulted in lower levels of Nfe2l2 mRNA, protein, and transactivating activity compared with scrambled siRNA. Nfe2l2 siRNA targeting Nfe2l2 mRNA was also confirmed by its trans-activating capacity of an ARE upstream from a luciferase reporter gene (ARE luciferase) in hepatocytes of mutant and control mice (Fig. 2C). We then investigated the consequences of Nfe2l2 knockdown for ROS accumulation in ApcHepKO and control mice hepatocytes using a small interfering (siRNA) strategy targeting Nfe2l2 mRNA. Transfection with Nfe2l2 siRNA resulted in lower levels of Nfe2l2 mRNA, protein, and transactivating activity compared with scrambled siRNAs, and lower levels of Nrf2 target gene expression at both transcript and protein levels in ApcHepKO hepatocytes (Fig. 3A-C). The specific Nrf2 target Nqo1 (NAD[P]H:quinone-oxidoreductase-1) then looked at their expression profiles and observed significant increases both at the transcriptional and protein levels in ApcHepKO mice.}

**FIG. 1.** Effects of pro-oxidative treatments on β-catenin-activated hepatocytes. (A) Left panels: Representative DHE staining of liver sections from control or ApcHepKO mice under normoxia (left) or after 24 hours of hypoxia (10% O2) (right) (n = 9 control and n = 9 ApcHepKO mice). Right panels: Nuclear Hoechst staining (left) and representative DHE staining of control or ApcHepKO hepatocytes cultured for 45 minutes under normoxia (center) or hypoxia (1% O2, right) (n = 4 control and n = 4 ApcHepKO mice). (B) Nuclear Hoechst staining (upper) and representative DHE staining of control or ApcHepKO hepatocytes (lower) cultured in the presence of 10 µM rotenone (left panels) or 5 mM paraquat for 3 hours (right panels) (n = 4 control and n = 4 ApcHepKO mice). (C) Left: H2O2 concentrations in control or ApcHepKO hepatocytes cultured with 10 µM rotenone, 10 µM rotenone, and 7.5 mM NAC or 5 mM paraquat for 3 hours. Right: Lipid peroxidation analysis; MDA quantification after paraquat treatment. The results are presented as means ± SEM from three independent experiments done on n = 3 control and n = 3 ApcHepKO mice. Significant differences are shown with asterisks *P < 0.05; **P < 0.01; ***P < 0.005.
Fig. 2. Nrf2 program induction in Apc<sup>HepKO</sup> livers. (A) Left and right panels: Relative levels of mRNA for ROS detoxification enzymes in control or Apc<sup>HepKO</sup> livers 6 days after injection of tamoxifen. The results are presented as means ± SEM (n = 12 control mice and n = 12 Apc<sup>HepKO</sup> mice). (B) Left panel: Immunoblots of cell extracts from control or Apc<sup>HepKO</sup> mice with antibodies specific for Nrf2, Nqo1, Gstm1/2, Keap1, and γ-tubulin. Right panels: Quantitative analysis of immunoblots. The results are presented as means ± SEM from three independent experiments (n = 6 control and n = 6 Apc<sup>HepKO</sup>). (C) Left panel: Immunoblot of nuclear extracts from control and Apc<sup>HepKO</sup> livers with an antibody specific for Nrf2. Right panel: Apc<sup>HepKO</sup> and control hepatocytes were transfected with the ARE-luciferase construct, and luciferase activity was assayed 24 hours after transfection. Results are expressed as the mean luciferase induction in three independent experiments carried out in triplicate.
displayed a strong decrease in expression after Nfe2l2 knockdown, whereas Gstm3 expression was only partially reduced (Fig. 3B).

Exposure to oxidative stress decreases the cellular GSH (reduced glutathione) to GSSG (oxidized glutathione) ratio, an index of cellular oxidative stress.
FIG. 3. Apc\textsuperscript{HepKO} hepatocytes constitutively produce ROS. (A-D) Apc\textsuperscript{HepKO} and control hepatocytes were transfected with the ARE-luciferase construct and either Si-Scramble or Si-Nfe2l2. (A) Luciferase activity was assayed 24 hours after transfection. Results are expressed as the mean luciferase induction for two independent experiments carried out in triplicate. (B) Relative levels of mRNA for Nfr2, Nqo1, and Gstm3 24 hours after transfection. (C) Immunoblot analysis of cell extracts from control or Apc\textsuperscript{HepKO} mice, with antibodies specific for Nfr2, Nqo1, and Gapdh. (D) Left: Glutathione analysis. Results are expressed as the ratio of reduced to oxidized glutathione, and the mean ± SEM from three independent experiments carried out in triplicate are shown (n = 3 control and n = 3 Apc\textsuperscript{HepKO} mice). Right: H2O2 concentrations in primary hepatocytes from control or Apc\textsuperscript{HepKO} mice 24 hours after transfection. Results are expressed as the mean of three independent experiments carried out in triplicate. (E) Apc\textsuperscript{HepKO} and control hepatocytes were cultured in the presence of halofuginone, an Nrf2 inhibitor. Upper left panel: Immunoblot of cell extracts from control or Apc\textsuperscript{HepKO} mice with antibodies against Nfr2 or Gapdh. Upper right panel: Representative DHE staining of control or Apc\textsuperscript{HepKO} cells after halofuginone treatment. Lower left panel: Lipid peroxidation analysis; MDA quantification after halofuginone treatment. Lower right panel: Immunoblot of γ-H2AX in Apc\textsuperscript{HepKO} and control cells after halofuginone treatment. Abbreviation: Gapdh, glyceraldehyde 3-phosphate dehydrogenase.

Total glutathione levels are unaffected by β-catenin activation (Supporting Fig. S1D). Following Nfe2l2 knockdown, the GSH/GSSG ratio decreased in both genotypes, but to significantly lower levels in Apc\textsuperscript{HepKO} cells, indicating a more reduced intracellular environment in these cells (Fig. 3D). We therefore investigated the effect of Nfe2l2 knockdown on ROS accumulation under basal conditions. Nfe2l2 knockdown increased H2O2 accumulation in Apc\textsuperscript{HepKO} cells, but not control cells (Fig. 3D). We also used halofuginone, known to inhibit Nrf2 accumulation pharmacologically.\textsuperscript{(24)} Although halofuginone may affect protein synthesis, protein yield and profile were not affected under our condition (Supporting Fig. S2A). Short-term treatment completely eliminated Nrf2 from both control and Apc\textsuperscript{HepKO} cells (Fig. 3E) and led to higher levels of ROS accumulation (Fig. 3E), resulting in higher levels of MDA accumulation (Fig. 3E) in Apc\textsuperscript{HepKO} cells and increased cell death (Supporting Fig. S2B). MDA causes DNA damage by forming adducts with DNA.\textsuperscript{(25)} We assessed the Ser139 phosphorylation of H2A histone family member X (γH2AX), a marker of DNA breaks. Halofuginone treatment led to higher levels of H2AX phosphorylation in Apc\textsuperscript{HepKO} cells than in controls (Fig. 3E and Supporting Fig. 2C). Thus, Apc\textsuperscript{HepKO} cells intrinsically generate more ROS than control cells, but keep them in check by directly activating Nfe2l2 expression and the Nrf2 pathway, to limit DNA damage. The genetic or pharmacological knockdown of Nrf2 is sufficient to trigger higher levels of ROS accumulation and oxidative damage in β-catenin-activated hepatocytes.

We then investigated the mechanisms by which Apc\textsuperscript{HepKO} hepatocytes had an activation of the Nrf2 pathway and asked whether the Nrf2 pathway activation associated with β-catenin activation was dependent on the enhanced ROS production. Thus, we pretreated mice with the ROS scavenger NAC in the drinking water for 3 weeks before an oxidative challenge. We first analyzed the expression of the antioxidant program. NAC treatment did not affect the Nrf2 program in the control mice, while it was greatly reduced in the mutant mice. Only Gstm1 and Gsta4, which are also β-catenin target genes,\textsuperscript{(23)} were not returned to basal level. In contrast, Nfe2l2 expression was significantly induced in the mutant mice regardless of the NAC treatment, consistent with the fact that Nfe2l2 is a direct β-catenin target gene.\textsuperscript{(20)} We then challenged the NAC pretreated hepatocytes with rotenone (10 µM for 3 hours) and assessed ROS accumulation in these cells. Although Apc\textsuperscript{HepKO} were protected against the oxidative stress induced by rotenone, this protection was partially lost when cells were pretreated with NAC (Fig. 4).

Altogether, these results indicate that the aberrant activation of the β-catenin signaling in Apc\textsuperscript{HepKO} results in an increased Nfe2l2 transcription, responsible for the elevated basal Nrf2 antioxidant program that promotes ROS detoxification and protects against oxidative damages.

**CONTRIBUTION OF Nfe2l2 TO THE METABOLIC PHENOTYPE OF β-CATENIN ACTIVATED HEPATOCYTES**

We previously demonstrated that hepatic β-catenin activation was associated with higher levels of mitochondrial respiratory activity.\textsuperscript{(17)} Higher levels of Nrf2 activity have also been shown to contribute to FAO rates.\textsuperscript{(26)} We therefore investigated the effects of pharmacological Nrf2 knockdown on...
Fig. 4. ROS production contributes to the enhanced Nfr2 pathway expression in Apc\textsuperscript{HepKO} hepatocytes. NAC was added to the drinking water of control and Apc\textsuperscript{HepKO} mice for 3 weeks. (A) Relative levels of mRNA for ROS detoxification enzymes in control or Apc\textsuperscript{HepKO} livers 6 days after injection of tamoxifen and 3 weeks of NAC or normal water. The results are presented as the means ± SEM (n = 8 control mice and 8 Apc\textsuperscript{HepKO} mice). (B) After 3 weeks of \textit{in vivo} NAC treatment, hepatocytes were isolated and cultured for 3 hours in the presence of 10 μM rotenone, and DHE staining was carried out (n = 4 control mice and n = 4 Apc\textsuperscript{HepKO} mice).
the metabolic activity of Apc\textsuperscript{HepKO} cells. Apc\textsuperscript{HepKO} cells had higher rates of CO\textsubscript{2} production from fatty acids than control cells, but the levels of CO\textsubscript{2} produced were significantly lower in the absence of Nrf2. Ketone body production was boosted, indicating that the end-product of FAO, acetyl-CoA, was mostly redirected to ketone bodies at the expense of the Krebs cycle in the absence of Nrf2 (Fig. 5A). Thus, overall, FAO was unaffected by the absence of Nrf2, but the fate of the acetyl-CoA generated was altered. However, treatment with an antioxidant, 10 µM butylated hydroxyanisole restored FAO to levels similar to those in untreated cells. Thus, quenching ROS overproduction was sufficient to abolish this effect (Fig. 5A). β-catenin activation leads to an increase in FAO, with Nrf2 redirecting part of the produced acetyl-CoA away from ketogenesis and toward the Krebs cycle, feeding the electron transfer chain and contributing to ROS production (Fig. 5B).

**HCCs EXPRESSING AN NRF2 SIGNATURE ARE ENRICHED IN CTNNB1 MUTATIONS**

Using the Cancer Genome Atlas (TCGA)–liver HCC data set (371 samples), we searched for a NRF2 signature recently described in human cancer. Using this NRF2 signature, we found that HCCs clustered into two groups: one including HCCs with high expression levels of most signature genes, and the second comprising HCCs that did not express the NRF2 signature. As expected, hierarchical clustering showed that most (14 of 19) of the HCC mutated for NFE2L2 or KEAP1 expressed a high NRF2 signature (Fisher’s exact test; $P < 0.0001$) (Fig. 6A).
More interestingly, we observed an enrichment of the CTNNB1 HCC in the positive NRF2 cluster, with 42 of 106 versus 49 of 265 in the negative NRF2 negative cluster (Fisher's exact test; $P < 0.0001$; odds ratio = 2.89) (Fig. 6A). Interestingly, patients with CTNNB1 HCC with a NRF2 signature showed a
significantly shorter survival (Gehan-Breslow generalized Wilcoxon method; \( P = 0.01 \)) compared with CTNNB1 HCC that did not express the NRF2 signature (Fig. 6B).

We also studied the correlation between the presence of a NRF2 signature and CTNNB1 mutations as well as other mutations. We found that CTNNB1 and TP53 were the most prevalent mutations in the positive NRF2 signature cluster (Supporting Fig. S3A).

The low frequency of NFE2L2 or KEAP1 mutations in HCC highlighted that the mechanisms of NRF2 activation were largely independent of the mutational status of the NRF2 pathway. Indeed, half of the NRF2-activated HCC were mutated for CTNNB1, showing that, in accordance with data from our Apc\textsuperscript{HepKO} mouse model, oncogenic \( \beta \)-catenin signaling was a main driver of NRF2-pathway activation in HCC (Fig. 6A).

To confirm the cross-talk between the \( \beta \)-catenin signaling and the NRF2 pathway, we analyzed, using the TCGA cohort data, the expression level of NFE2L2, KEAP1, and three canonical target genes of the oxidative stress pathway: NQO1, TXN, and TXNRD1 (Fig. 6C). We observed a significant increase of NFE2L2 in CTNNB1-mutant HCC, regardless of the NRF2 signature, which is consistent with the fact that NFE2L2 is a target of the \( \beta \)-catenin signaling\(^{20}\) (Fig. 6C). Moreover, we observed a significant increase of KEAP1 in the HCC that have a positive NRF2 signature (Supporting Fig. S3B). This is consistent with the fact that KEAP1 is a target of the NRF2 pathway as part of a negative feedback loop\(^{27}\) and agreed with the results presented on Fig. 6A. However, it was the case only for HCC nonmutated for CTNNB1, named CTNNB1 WT. The reason why this was not observed in CTNNB1-mutant HCC remains to be investigated (Supporting Fig. S3B).

The activation of the NRF2 pathway in CTNNB1 HCCs is counterintuitive with the co-occurrence of mutations in CTNNB1 with either NFE2L2 or KEAP1, although these events were infrequent\(^{28}\). We analyzed the gene-expression levels of three NRF2 target genes involved in the antioxidant program (NQO1, TXN, and TXNRD1) in HCC bearing a single CTNNB1 mutation compared with HCC having mutations in both CTNNB1 and components of the NRF2 pathway, but could not detect any significant difference (Fig. 6C).

**TARGETING REDOX BALANCE FOR THERAPEUTIC EFFECTS ON CTNNB1 HCC**

We assessed the potential therapeutic effect of targeting redox balance in CTNNB1 HCC using the previously described \( \beta \)-catenin-activated HCC mouse model (hereafter named Apc HCC).

Similar to in CTNNB1 HCC, Apc HCC tumors displayed an Nrf2 program (Fig. 7A). Despite the presence of hypoxic zones in Apc HCC (Supporting Fig. S4A), we detected no abnormal ROS accumulation on DHE-stained tumor sections (Fig. 7A). In preneoplastic Apc\textsuperscript{HepKO} cells, Nfe2l2 knockdown was sufficient to generate levels of ROS accumulation and toxic byproducts, such as lipid peroxides, higher than in normal cells. An unbalanced ROS/AOD ratio can therefore be deleterious to cells, leading to DNA damage beyond the threshold for cell death. We therefore looked at the consequences of a direct inhibition of Nrf2 activity on \( \beta \)-catenin-activated HCC development, using our mouse model.

All Apc HCCs had high levels of nuclear \( \beta \)-catenin accumulation (Supporting Fig. S4B). Following detection of the first tumors, the animals received a daily injection of 250 \( \mu \)g/kg halofuginone, and...
tumor progression was followed weekly, by echography. After 3 weeks of treatment, the tumor growth rate of halofuginone-treated tumor-bearing mice was less than 1% that of controls; the pharmacological inhibition of Nrf2 therefore effectively blocked β-catenin-induced HCC development. We also
investigated another therapeutic approach based on the control, by β-catenin signaling, of expression of the cytochrome P450 family member Cyp2e1\(^{29,30}\) (Fig. 2A), which is involved in detoxifying acetaminophen (APAP). APAP is metabolized by Cyp2e1 to generate \(N\)-acetyl-\(p\)-benzoquinoneimine, which forms adducts with glutathione, leading to deleterious ROS accumulation.\(^{31}\) β-catenin knockout mice are protected against APAP intoxication, providing a direct link between APAP catabolism and β-catenin activity.\(^{32}\) Low doses of APAP (0.1 mM) generates ROS accumulation and are toxic in cells with high levels of Cyp2e1, such as cells with high β-catenin activity, but not in normal cells with lower levels of Cyp2e1 (Supporting Fig. S4C). We first injected a single dose of 300 mg/kg APAP into control and Apc\(^{HepKO}\) mice. Control mouse livers were not affected by this low dose (Fig. 6C), whereas Apc\(^{HepKO}\) mouse livers presented large areas of lysis and high plasmatic level of alanine aminotransferase activity, confirming the liver damages (Fig. 6C).

We investigated the effects of APAP treatment on Apc HCC. Once the first tumors were detected, control mice and mice bearing β-catenin HCC tumors received a single 300-mg/kg APAP injection, and tumor growth was followed weekly, by echography. This injection led to a marked decreased in the tumor growth rate of HCC tumors with β-catenin activation (Fig. 7D).

**Discussion**

This study revealed cooperation between the NRF2 pathway and oncogenic β-catenin during CTNNB1-mediated HCC tumorigenesis. We found that (1) β-catenin activation triggers a pro-oxidative state and increases the expression of \(N\)fe2l2, which elevates the basal Nrf2 antioxidant program that promotes the ROS detoxification program and tumorigenesis; (2) Nrf2 activation contributes to the metabolic profile of β-catenin activated hepatocytes in mice; (3) CTNNB1 HCCs have an increased expression of \(N\)fe2l2, which resulted in half of them in the presence of an NRF2 signature largely independent of \(NFE2L2\) or KEAP1 mutations; and (4) the detoxification pathway is a suitable therapeutic target, as treatments targeting either the Nrf2 pathway or the glutathione pool blocked the development of β-catenin-induced HCCs.

Apc\(^{HepKO}\) mutant mice, in which Apc is specifically inactivated in hepatocytes, results in an aberrant and constitutive activation of the β-catenin-signaling model CTNNB1 HCC.\(^{18,20,33,34}\) In this work, we showed that in Apc\(^{HepKO}\) mutant mice, β-catenin activation led to an increase in \(N\)fe2l2 mRNA levels in hepatocytes protecting these cells from the oxidative damage induced by the oncogenic signaling. The increase in \(N\)fe2l2 mRNA level is likely transcriptional, as \(N\)fe2l2 is a direct β-catenin target gene.\(^{20}\) Moreover, transcription of \(N\)fe2l2 is also regulated by the transcription factor Ahr, which is also a direct β-catenin target in the liver.\(^{35}\) Thus, as observed for other oncogenes, such as K-Ras\(^{G12D}\) or B-Raf\(^{V61E}\),\(^{36}\) the oncogenic β-catenin signaling induced \(N\)fe2l2 transcription to promote an Nrf2 antioxidant program, which led to ROS detoxification and tumorigenesis. Indeed, inhibition of the Nrf2 program prevented β-catenin-induced HCC development. We have therefore identified that β-catenin cooperates with the Nrf2 pathway to promote HCC tumorigenesis. We did not totally clarify the mechanism by which Nrf2 activity promotes β-catenin-induced HCC tumorigenesis, but our data showed that it is at least through the maintenance of the redox homeostasis that is required for the survival of the Apc\(^{HepKO}\) hepatocytes, but other mechanisms are likely to be also involved.
Metabolic reprogramming in cancer cells is tightly associated with ROS production. The major metabolic feature of tumors with β-catenin activation is enhanced FAO and ketone production. The Nrf2 pathway has been shown to enhance FAO in various tissues. However, in the liver we show that it mostly redirects FAO-derived acetyl-CoA to the Krebs cycle at the expense of ketogenesis, therefore increasing the pool of NADH and FADH₂ feeding the ETC. This finding is consistent with observations that cells with constitutive Nrf2 activity have a higher rate of adenosine triphosphate (ATP) production by ATP synthase, as we previously reported for Apc<sup>HepKO</sup> cells. Contrary to previous reports, we found that in the liver, antioxidant treatment, in absence of Nrf2, was sufficient to rescue CO₂ production, indicating that ROS probably affect ETC efficiency through oxidation of several proteins of the ETC complexes. In β-catenin-activated hepatocytes, PPARα activation triggers higher FAO rates, but the increase in Nrf2 pathway activity, by quenching excess ROS, will help to feed the ETC efficiently and increase ATP production. Thus, the Nrf2 pathway contributes to the metabolic oxidative program in the Apc<sup>HepKO</sup> mouse model, but it also confers survival advantages through its cytoprotective effect.

Constitutive activation of the Nrf2 pathway due to gain-of-function NFE2L2 mutations or loss-of-function KEAP1 mutations has been observed in various cancers, indicating a need for these cells to keep their ROS levels in check. However, the overall frequency of these mutations remains low (below 2%) over a large spectrum of tumors, with considerable variability between cancer types, the frequency being highest in non–small-cell lung carcinomas. It has been shown that 6% of HCCs carry NFE2L2 or KEAP1 mutations, and that HCCs have the second highest levels of Nrf2 program activity of diverse tumors, suggesting that these tumors used another route for constitutive Nrf2 activation. Our data showed that it was indeed the case. We found that nearly half of the CTNNB1 HCC revealed a positive Nrf2 signature. However, only half of the CTNNB1 HCC revealed a positive Nrf2 signature. The reason why it is the case remains to be investigated, but it illustrates the complexity of the Nrf2-driven transcription that is required to maintain a metabolic reprogramming able to support the tumor development. We also searched whether occurrence of CTNNB1 mutations with mutations in either KEAP1 or NFE2L2 could confer a better antioxidative protection to tumoral hepatocytes, but our analyses of NQO1, TXN, or TXNRD1 did not support this hypothesis.

Many chemotherapy and radiotherapy treatments rely on the generation of high levels of ROS to kill cancer cells. Conversely, Nrf2 activity, by inducing drug-metabolizing enzymes, redox-modulating proteins and drug exporters, contributes to enhanced chemoresistance. Targeted treatments that aim to decrease Nrf2 levels in conjunction with chemotherapy have been investigated, and several Nrf2 inhibitors have been developed and tested. One of these compounds, halofuginone, is considered particularly promising, as it inhibits Nrf2 activity in therapy-resistant cancer cells with constitutive Nrf2 activation and increases the efficiency of cisplatin or doxorubicin treatment. These inhibitors are subject to the limitations of use generally applying to cancer treatments. They do not eliminate all cancer cells, but they do eliminate some normal cells. Consistent with a previous study, we used a therapeutic approach based on the control, by β-catenin signaling, of Cyp2e1 expression. Using APAP to deplete the glutathione pool specifically in cells with CTNNB1 mutations, it should be possible to bypass the existing Nrf2 protection program, given that low doses of APAP have no effect on the surrounding healthy tissues. HCC mostly occurs in cirrhotic livers (80%), raising questions about the use of APAP in such a poorly viable environment, but many HCCs with CTNNB1 mutations nevertheless occur in the absence of cirrhosis, and the use of APAP, which has been prescribed as an over-the-counter analgesic for the last 60 years, could be considered in such cases.

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Author names in bold designate shared co-first authorship.

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