**SUMMARY**

Unlike human and rat hepatocellular carcinoma, chemically induced mouse hepatocellular carcinomas do not show Nrf2 mutation/activation. Furthermore, metabolic reprogramming of neoplastic cells is absent as well. The results suggest that the mouse is not the ideal model to investigate the role of nuclear factor (erythroid-derived 2)-like 2 in hepatocarcinogenesis.

**BACKGROUND & AIMS:** Activation of the kelch-like ECH-associated protein 1 (Keap1)–nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway has been associated with metabolic reprogramming in many tumors, including hepatocellular carcinoma (HCC). However, the contribution of Nrf2 mutations in this process remains elusive. Here, we investigated the occurrence of Nrf2 mutations in distinct models of mouse hepatocarcinogenesis.

**METHODS:** HCCs were generated by experimental protocols consisting of the following: (1) a single dose of diethylnitrosamine (DEN), followed by repeated treatments with the nuclear-receptor agonist 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene; (2) repeated treatments with 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene alone; (3) a single dose of DEN followed by exposure to a choline-deficient L-amino acid–defined diet; and (4) a single dose of DEN with no further treatment. All of these protocols led to HCC development within 28–42 weeks. Activation of the Keap1-Nrf2 pathway was investigated by analyzing the presence of Nrf2 gene mutations, and the expression of Nrf2 target genes. Metabolic reprogramming was assessed by evaluating the expression of genes involved in glycolysis, the pentose phosphate pathway, and glutaminolysis.

**RESULTS:** No Nrf2 mutations were found in any of the models of hepatocarcinogenesis analyzed. Intriguingly, despite the described cooperation between β-catenin and the Nrf2 pathway, we found no evidence of Nrf2 activation in both early dysplastic nodules and HCCs, characterized by the presence of up to 80%–90% β-catenin mutations. No HCC metabolic reprogramming was observed either.

**CONCLUSIONS:** These results show that, unlike rat hepatocarcinogenesis, Nrf2 mutations do not occur in 4 distinct models of chemically induced mouse HCC. Interestingly, in the same models, metabolic reprogramming also was minimal or absent, supporting the concept that Nrf2 activation is critical for the switch from oxidative to glycolytic metabolism.
Keywords: Nrf2; Ctnnb1; Gene Mutation; Metabolic Reprogramming; HCC.

The knowledge of the genetic/epigenetic alterations implicated in hepatocellular carcinoma (HCC), one of the leading causes of cancer-related deaths worldwide, is still fragmentary. Among the several modifications involved in HCC development, those involving Nrf2 (encoded by NFE2L2) are of particular interest, because the role of the kelch-like ECH-associated protein 1–nuclear factor (erythroid-derived 2)-like 2 (KEAP1–NRF2) pathway in cancer progression has provided conflicting results.2–4 Nrf2 is a master transcriptional activator of genes encoding enzymes that protect cells from oxidative stress and xenobiotics, and of various drug efflux pump members of the multidrug resistance protein family.5 Nrf2 is negatively regulated and targeted to proteasomal degradation by KEAP1.6–9 Several studies have reported that point mutations in KEAP1 or NRF2 genes often are present in primary tumors.10–15 Regarding human HCC, 2 studies performing whole-exome sequencing have shown mutations of either Nrf2 (6.4%) or KEAP1 (8%), suggesting that the dysregulation of this pathway may play a relevant role in a subset of human HCCs.16–18 Notably, increased levels of Nrf2 messenger RNA (mRNA) have been found to be associated with poor prognosis in human HCC.19 Although activation of this pathway has been shown in a variety of models of rodent hepatocarcinogenesis, the impact of Nrf2 mutations on this process remains elusive. Indeed, although a striking incidence of gene mutations has been found in preneoplastic and neoplastic rat hepatocytes,20–22 in mouse models the oncogenic role of Nr2f was instead attributed to its increased nuclear translocation and transcriptional activity, as a consequence of the accumulation of p62 it is binding to Keap1, and consequent release of Nrf2.23–26

This study aimed to investigate whether Nrf2 mutation/activation occurs in distinct mouse models of hepatocarcinogenesis. In addition, because it recently was reported that in human HCCs characterized by β-catenin mutations the KEAP1–NRF2 pathway is activated independently from mutations of these genes,27,28 we also wished to determine whether Nrf2 mutations could co-occur in β-catenin mutated HCC. Finally, we also investigated whether metabolic reprogramming, a hallmark of cancer cells, takes place in these experimental models. The results showed the complete absence of Nrf2 mutations in all the examined murine models of hepatocarcinogenesis. They also indicated that the Keap1–Nrf2 pathway is not activated in Ctnnb1 mutated HCCs, as well as in other models of mouse HCC. Finally, the analysis of the expression of genes involved in glycolysis, pentose phosphate pathway (PPP), and glutamine metabolism did not show any clear evidence of metabolic reprogramming.

Abbreviations used in this paper: CDAA, choline-deficient L-aminocitc acid–defined diet; cDNA, complementary DNA; DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; Keap1, kelch-like ECH-associated protein 1; mRNA, messenger RNA; Nr2f, nuclear factor (erythroid-derived 2)-like 2; PPP, pentose phosphate pathway; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene.

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Results
Nrf2 Mutations Are Absent in HCCs Generated by Diethylnitrosamine + 1,4-Bis-[2-(3,5-Dichloropyridyloxy)]Benzene or 1,4-Bis-[2-(3,5-Dichloropyridyloxy)]Benzene Alone

Previous studies have shown that a single treatment with diethylnitrosamine (DEN) followed by repeated treatments with 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), the most potent agonist of constitutive androstane receptor and a nongenotoxic mouse hepatocarcinogen, or TCPOBOP alone induced HCC development in 100% of mice and that 81% and 90% of HCCs, respectively, showed Ctnnb1 mutations.29 Chronic treatment with TCPOBOP is associated with liver dysplasia and enhanced oxidative stress, although the proteins responsible for the increased oxidative stress have yet to be identified.30 Because activation of Nrf2 represents one of the major antioxidant defense mechanisms leading to increased transcription of several cytoprotective enzymes and enhanced cell survival,6,9 we investigated whether activation of the Keap1–Nrf2 pathway could be involved in HCC development in mice.

Our previous studies have shown that rat preneoplastic and neoplastic lesions induced by protocols of chemical hepatocarcinogenesis have a very high incidence of Nrf2 mutations.20–22 Therefore, we initially investigated the presence of gene mutations in laser-microdissected mouse HCCs developed 28 weeks after a single dose of DEN followed by repeated treatments with TCPOBOP (experimental protocol 1). Microscopically, these tumors showed features typical of HCC such as atypical nuclei, cellular pleomorphism, and mitoses, as well as cell death (Figure 1A). Selected areas of livers from mice treated with TCPOBOP alone or untreated mice also were included in the analysis as control groups. Because published works indicate that most of the Nrf2 mutations occur in exon 2,31 we sequenced this exon in 20 HCCs using Sanger fluorescence-based sequence analysis. Quite unexpectedly, no mutation of Nrf2 was observed in any of the 20 examined HCCs generated by the treatment consisting of DEN + TCPOBOP (Table 1), as well as in control livers or livers from mice treated with TCPOBOP alone for 28 weeks.

Next, we analyzed Nrf2 mutations in HCCs generated after 42 weeks of repeated treatment with TCPOBOP in the absence of DEN (experimental protocol 2). Similar to what was observed in HCCs obtained with the DEN + TCPOBOP
protocol, these tumors were characterized by nuclear atypia, pleomorphism, and increased mitotic activity (Figure 2A). As shown in Table 1, no Nrf2 mutation was found in these HCCs.

These results indicate that unlike preneoplastic and neoplastic lesions developed in rat livers after DEN treatment, mouse HCCs generated by the 2 aforementioned protocols are completely devoid of Nrf2 mutation.

**The Keap1–Nrf2 Pathway Is Not Activated in β-Catenin Mutated HCCs**

Recent work on the mouse and human HCCs characterized by β-catenin mutations has shown that the Keap1–Nrf2 pathway is activated independently from Nrf2 mutations, suggesting cooperation between the oncogenic β-catenin and Nrf2 pathways in Ctnnb1-mediated HCC tumorigenesis.27,28 Because HCCs generated by DEN + TCPOBOP or TCPOBOP alone display from 80% to 90% of Ctnnb1 mutations,29 we wished to assess whether activation of the Keap1–Nrf2 pathway could occur concomitantly with β-catenin mutation, but independently from Nrf2 mutation.

Because sustained activation of the Keap1–Nrf2 pathway in the absence of Nrf2 mutations already has been reported in animal models of rat hepatocarcinogenesis,29,30 we examined such a possibility in tumors induced by DEN + TCPOBOP or TCPOBOP alone. To verify the status of the Keap1–Nrf2 pathway, we analyzed the mRNA profiling of these HCCs and their respective controls. As previously reported,29 hierarchical clustering analysis of gene expression patterns performed on these HCCs did not identify any significant difference between HCCs generated by a genetic agent DEN and those caused by repeated administration of TCPOBOP alone. With regard to the Keap1–Nrf2 pathway, functional analysis of differentially expressed genes did not identify the Nrf2-mediated oxidative stress response among the most affected pathways. Moreover, when Ingenuity Pathway Analysis (IPA) was applied to investigate the Nrf2 target genes, the results showed that the expression of most of the Nrf2 target genes were not affected or even down-regulated (Figures 1B and C and 2B and C).

Next, to validate the transcriptomic profiling data we performed quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis on the same complementary DNA (cDNA) used for gene sequencing. The results showed that among the examined Nrf2 target genes (Nqo1, Hmox1, Gclc, Gsta4), Gsta4 was the only one significantly up-regulated in tumors compared with control livers (Figure 1D). Along the same line, no significant change in the expression of any of the same Nrf2 target genes was observed in HCCs generated by repeated treatment with TCPOBOP in the absence of DEN (Figure 2D). Lack of Nrf2 activation in mouse HCCs was confirmed by immunohistochemical analysis of NAD(P)H dehydrogenase quinone 1 (NQO1), the best known target gene of Nrf2, showing that neoplastic mouse hepatocytes were completely negative (Figures 1E and 2E). Conversely, intense NQO1 immunostaining was observed in rat hepatocytes from nodules previously shown to carry Nrf2 mutation and activation of the Keap1–Nrf2 pathway (Figure 1F).20

**The Keap1–Nrf2 Pathway Is Not Activated in β-Catenin Mutated Dysplastic Nodules**

Our previous studies reported that activation of the Keap1–Nrf2 pathway occurs as early as in preneoplastic

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### Table 1. Analysis of Nrf2 Mutations in Different Experimental Settings

| Treatment        | Strain | Sex | Time of death | number of Tumors | Number of Nrf2 mutations | Number of Ctnnb1 mutations |
|------------------|--------|-----|---------------|------------------|--------------------------|--------------------------|
| DEN + TCPOBOP    | C3H    | F   | 28 weeks      | 20               | 0                        | 81% (Ref. 29)            |
| TCPOBOP          | C3H    | F   | 42 weeks      | 11               | 0                        | 90.9% (Ref. 29)          |
| DEN + CDAA       | C57BL  | M   | 25 weeks      | 10               | 0                        | ND                       |
| DEN              | B6C3F1 | F   | 40 weeks      | 11               | 0                        | ND                       |

CDAA, choline-deficient L-amino acid–defined diet; DEN, diethylnitrosamine; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene;

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**Figure 1.** (See previous page). Histologic and molecular analyses of HCCs of DEN + TCPOBOP-treated mice. (A) Microphotographs showing HCCs generated in mice treated with a single dose of DEN, followed by repeated treatment with TCPOBOP and killed 28 weeks thereafter (H&E: left, ×20; right, ×40). (B) Enriched functional pathways in HCCs vs TCP-treated livers. P values were determined using the Ingenuity scoring system. (C) Nrf2-mediated oxidative stress response pathway. Red, up-regulation; white, not modified; grey, no expression; yellow, genes whose expression was modified but did not pass the P<0.05. (D) qRT-PCR analysis of NAD(P)H dehydrogenase quinone 1, Hmox1, Gclc, and Gsta4 mRNA levels in 15 HCCs of mice treated as in panel A. In the HCC group, each dot corresponds to 1 tumor analyzed. Gene expression is reported as fold change compared with controls. The endogenous control gene mouse Gapdh was used for normalization. The results are expressed as means + SD. *P < .05. (E) Representative image of a mouse HCC induced as in panel A. (F) Representative image of a rat preneoplastic nodule generated by the R-H model,21 both stained with antibody anti-NAD(P)H dehydrogenase quinone 1 (left, ×20; right, ×5). AHR, Aryl hydrocarbon receptor; AKT, Akt serine/threonine kinase family; ASK1, Apoptosis signal-regulating kinase 1; CAR, Constitutive androstane receptor; CTRL, control; DENA, Diethylnitrosamine; ERK, Extracellular signal-regulated kinases; IL, interleukin; LPS, lipopolysaccharide; LXR, Liver X receptor; MAPK, Mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MEKK, Mitogen-activated protein kinase 1; PI3K, Phosphoinositide 3-kinase; PKC, Protein kinase C; PXR, Pregnane X receptor; Ras, Rat sarcoma; ROS, reactive oxygen species; RXR, Retinoid X receptor; TAK1, Mitogen-activated protein kinase 7; TCP, TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene).
nodules induced in rats by protocols of chemical hepatocarcinogenesis. To rule out the possibility that the Nrf2 signaling pathway is activated during the early phase of tumorigenesis in mice, we investigated the Keap1–Nrf2 pathway in low- and high-grade dysplastic nodules generated 21 weeks after a single dose of DEN followed by...
weekly injections of TCPOBOP. At this time point, the earliest neoplastic lesions are identifiable in the liver of mice subjected to this protocol. For this purpose, we analyzed the expression of 3 well-known Nrf2 target genes (Nqo1, Gclc, and Gsta4). As already reported, 13 of 16 (81%) of these dysplastic nodules showed Ctnnb1 mutation. The results of qRT-PCR analysis did not show any clear evidence of activation of the Nrf2 pathway. Indeed, although Nqo1 expression was enhanced compared with control liver, albeit at a very low level, no significant change was observed for Gst4a and Gclc (Figure 3A–C). The lack of activation of the Nrf2 pathway was confirmed further by immunohistochemistry analysis, which showed a lack of Nqo1 staining in both low- and high-grade dysplastic nodules identified by their positivity to glutamine synthetase (Figure 3D and E).

Metabolic Reprogramming Does Not Occur in Mouse HCCs Subjected to DEN + TCPOBOP or TCPOBOP Alone

Nrf2 not only maintains redox homeostasis in quiescent cells, but also stimulates glucose consumption, PPP, and promotes the glutamine-utilizing reactions in the post–ribose-5-phosphate steps all suggestive of the metabolic reprogramming often associated with cancer cells. However, whether Nrf2 activation is a sine qua non condition for metabolic reprogramming is unknown. Therefore, we wished to determine whether metabolic reprogramming was associated with neoplastic development in tumors lacking Nrf2 activation. Because enhanced glycolysis and activation of PPP are a common feature of cancer cells, we investigated whether the expression of Hmox1, Gclc, and Gsta4 in mouse livers showing low-grade (upper panel) and high-grade (lower panel) dysplastic nodules positive for glutamine synthetase (GS) and negative for NQO1 immunostaining (×5). CTRL, control; TCP, TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene).

**Figure 3.** qRT-PCR analysis of Nqo1, Gclc, and Gst4a in dysplastic nodules. (A–C) qRT-PCR analysis of Nqo1, Gclc, and Gst4a in 14 dysplastic nodules of mice subjected to a single dose of DEN, followed by repeated treatments with TCPOBOP and killed 21 weeks thereafter. In the dysplastic nodules group, each dot corresponds to 1 nodule analyzed. Gene expression is reported as fold change relative to livers from untreated mice. Results are expressed as means ± SD. **P < .01. (D and E) Serial sections of mouse liver showing low-grade (upper panel) and high-grade (lower panels) dysplastic nodules positive for glutamine synthetase (GS) and negative for NQO1 immunostaining (×5). CTRL, control; TCP, TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene).
Figure 4. qRT-PCR analysis of G6pc, Glut1, Hk2, G6pdx, Mct4, and Glu in HCCs of mice treated with DEN and TCPOBOP. (A–E) qRT-PCR analysis of G6pc, Glut1, Hk2, G6pdx, and Mct4 in 15 HCCs of mice treated with a single dose of DEN, followed by repeated treatment with TCPOBOP and killed 28 weeks after DEN treatment. In the HCC group, each dot corresponds to 1 tumor analyzed. Gene expression is reported as fold change relative to livers from untreated mice. Results are expressed as means ± SD. *P < .05. (F) qRT-PCR analysis of Glu in HCCs from mice treated as in panel A. Gene expression is reported as fold change relative to livers from untreated mice. Results are expressed as means ± SD. (G) Immunohistochemical analysis of a mouse HCC. (H) Immunohistochemical analysis of a rat preneoplastic nodule stained with an antibody antiglutaminase (left, ×20; right, ×5). CTRL, control; DEN, Diethylnitrosamine; GLS, glutaminase; TCP, TCPOBOP (1,4-bis-[2-(3,5-dichloropyridylo)xy]benzene).
the glucose transporter 1 (Glut1), hexokinase 2 (Hk2), glucose-6 phosphate dehydrogenase (G6pdx) (the key limiting enzyme of the oxidative branch of PPP), and Mct4 (Slc16a3, responsible for lactate extrusion) were altered in HCCs induced by DEN + TCPOBOP. Because Nrf2 also is involved in glutamine metabolism and glutaminolysis, we determined the expression levels of glutaminase (Gls). Finally, we also examined the expression of G6pc, the enzyme that catalyzes the hydrolysis of glucose-6-phosphate to glucose in the terminal step of glycogenolysis and whose deficiency leads to increased glycolysis. As shown in Figure 4A–E, only the expression of Glut1, but not that of Hk2, G6pdx, G6pc, or Mct4, was up-regulated significantly in HCCs generated by DEN + TCPOBOP. No change in the mRNA levels of Glut1, Hk2, and Mct4 or G6pdx was found in tumors treated with TCPOBOP alone, although a significant decrease of G6pc mRNA levels was observed (Figure 5A–E).

Regarding glutaminolysis, we found that despite the increased expression of glutamine synthase previously found in HCC that developed in mice treated with DEN + TCPOBOP or TCPOBOP alone, no change in the expression of mRNA levels of Gls, the enzyme generating glutamate from glutamine, was observed in HCCs generated by both of these experimental protocols (Figures 4F and 5F). Accordingly, no enhanced protein expression was found in neoplastic mouse hepatocytes by immunohistochemistry (Figure 4G), whereas Gls was strongly enhanced in rat preneoplastic hepatocytes (Figure 4H).

| Figure 5. qRT-PCR analysis of G6pc, Glut1, Hk2, G6pdx, and Mct4 in HCCs of mice treated with TCPOBOP alone. (A–E) qRT-PCR analysis of G6pc, Glut1, Hk2, G6pdx, and Mct4 in 11 HCCs of mice subjected to repeated treatments with TCPOBOP and killed 42 weeks thereafter. Gene expression is reported as fold change relative to livers from untreated mice. Results are expressed as means ± SD. ***P < .001. (F) qRT-PCR analysis of Gls in 11 HCCs of mice treated as in panel A. In the HCC group, each dot corresponds to 1 tumor analyzed. Gene expression is reported as fold change relative to livers from untreated mice. Results are expressed as means ± SD. CTRL, control; TCP, TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene). |
Overall, these results indicate that metabolic reprogramming involving a shift toward glycolysis, PPP activation, and glutaminolysis does not take place in mouse HCCs that developed in these animal models.

**Nrf2 Mutations Are Absent in HCCs Generated by DEN + Choline-Deficient L-Amino Acid-Defined Diet**

We envisaged the possibility that the lack of Nrf2 mutations observed in mouse HCCs treated with TCPBP only could be owing to some as yet unknown inhibitory effect of TCPBP on Nrf2-mutated hepatocytes. To test this hypothesis, we scored the presence of Nrf2 mutations in 10 HCCs generated by a single dose of DEN followed by feeding a choline-deficient L-amino acid-defined diet (CDAA) for 25 weeks (experimental protocol 3); notably, in rats the same regimen led to a high frequency of Nrf2 mutations, ranging from 90% at the early stage of tumorigenesis to 25% in HCCs.22 However, unlike rat HCCs, no Nrf2 mutations could be observed in any of the examined murine tumors generated by a single dose of DEN followed by feeding a CDAA diet (Table 1).

**The Keap1–Nrf2 Pathway Is Not Activated in HCCs From Mice Subjected to DEN + CDAA**

To investigate whether Nrf2 activation still could take place in mouse HCCs generated by this protocol of hepatocarcinogenesis (Figure 6A), we analyzed the expression of Nrf2-target genes. As shown in Figure 6B–E, no change of the expression of the 4 examined Nrf2 target genes (Nqo1, Hmox1, Gclc, or G6pdx) was detected in the tumors, suggesting that the Keap1–Nrf2 pathway is not activated and...
Figure 7. Frequency of Nrf2 mutations in human beings, rats, and mice.

therefore it does not exert a pivotal role in chemically induced mouse HCC.

**Nrf2 Mutations Are Absent in HCCs Obtained by a Single Dose of DEN**

It was reported that although Nrf2 K0 mice are completely refractory to DEN-induced HCC, tumors developed in wild-type mice by a single dose of DEN in the absence of any promoting procedure alone display 100% Nrf2 mutation. To rule out that promoting procedures of a different nature (TCPOBOP or CDAA) could somehow interfere with the expansion of Nrf2 mutated cells, we scored mutations of this gene in mice in which HCC was induced by a single dose of DEN to weanling mice, without any further treatment (experimental protocol 4). However, none of the 11 HCCs induced by this protocol showed Nrf2 gene mutations (Table 1).

**Discussion**

The Keap1–Nrf2 axis is a redox-sensitive signaling system, regulating up to 10% of human genes. Activation of NRF2 not only protects cells from oxidative stress and DNA-damaging electrophiles, but it also confers cytoprotection against high levels of reactive oxygen species, leading to enhanced survival and resistance of cancer cells to chemotherapy. The findings that NRF2 overexpression and/or somatic mutations of this gene take place in many human cancers, including HCC, and that cancers with high NRF2 levels are associated with poor prognosis, have unveiled an oncogenic role of NRF2. Further support to the oncogenic role of NRF2 stems from the discovery that the genetic inactivation of Nrf2 is sufficient to impair liver tumorigenesis in rats and mice.

Among the several mechanisms responsible for Nrf2 activation, gene mutations have been described in approximately 6% of human HCCs, and in rats, since very early steps of the hepatocarcinogenic process, at a very high incidence, suggesting their critical role in HCC development. Notably, in human HCC, NRF2 mutations have been reported to occur concomitantly with β-catenin mutations. In support of cooperation between the Keap1–Nrf2 pathway and β-catenin signaling, a very recent article reported an enrichment of the NRF2 program in human HCCs with β-catenin (CTNNB1) mutations, largely independent of NRF2 or KEAP1 mutations. The same study also showed that mice with hepatocyte-specific oncogenic β-catenin activation increased Nrf2 activation, most likely to protect β-catenin–activated hepatocytes from oxidative damage, thus favoring tumor development, and proposed cooperation between oncogenic β-catenin signaling and the NRF2 pathway in CTNNB1-mediated HCC. Furthermore, an independent work showed co-activation of β-catenin and NRF2 in 9% of all human HCCs, and discovered that co-expression of mutated CTNNB1 with mutant NRF2, but not wild-type NRF2, led to rapid HCC development and mortality. Thus, it appears that the frequency of Nrf2 mutations greatly vary among HCCs occurring in different species (Figure 7).

Quite surprisingly, the results of the present work showed a complete lack of Nrf2 gene mutations in liver mouse tumors generated by 4 different experimental protocols. This finding was particularly unexpected because DEN, the chemical used in the present study to induce HCC, is a genotoxic carcinogen that, in rats, induces Nrf2 gene mutation in 90% of preneoplastic lesions and in 25% of HCCs. In this context, it should be noted that NRF2 mutations in rats occur in HCCs generated by a regimen almost identical to the experimental protocol 3 of the present study, consisting of a single dose of DEN followed by a choline-deficient diet. Furthermore, in light of the recent works showing cooperation between β-catenin and Nrf2 in promoting liver cancer, it is even more puzzling to find a complete absence of Nrf2 mutation and/or activation of this transcription factor in HCCs developed by DEN + TCPOBOP or TCPOBOP alone that displayed up to 80%–90% of mutations of Ctnnb1.

The reason why DEN-induced Nrf2 mutations confer a selective advantage to initiated hepatocytes in rat, but not mouse, liver is unknown. Metabolic activation of DEN to its carcinogenic electrophiles, generation of promutagenic lesions in DNA (ie, O6-EtG), and their fixation into daughter cells, are similar in both species, as shown by the high incidence of HCC induced in both rats and mice. Nevertheless, the type of mutation caused by DEN in mouse tumors greatly varies depending on genetic background, age, sex, and other factors. Indeed, although the majority of liver tumors generated by a single dose of DEN are mutated in either the Ha-ras or the B-raf gene, the prevalence of Ha-ras mutated tumors was significantly higher in the susceptible C3H and B6C3F1 mouse strains (39%–50%) than in the comparatively resistant C57BL mouse (7%). In contrast, B-raf mutated tumors were more frequent in C57BL mice (68%) than in the other 2 strains (17%–45%). It is also worth mentioning that Ha-Ras and B-raf mutations are rare in human HCCs, whereas more relevant similarities with human HCC are shown by liver tumors generated by mouse experimental models consisting of a single application of DEN followed by promoting
procedures, such as TCPOBOP or phenobarbital; indeed, these tumors display 80%--90% of Ctnnb1 mutations,29,42 a condition resembling human HCC in which mutations of this gene were among the most frequent (32.8%).16 Our present study showing a complete lack of Nrf2 mutations in 52 HCCs generated with 4 different models of mouse hepatocarcinogenesis, 3 distinct strains, both sexes, different age of the animals, and different doses of DEN, suggests that Nrf2 mutations are a rare event in mouse HCC, possibly because they do not provide any selective growth advantage to chemically induced initiated mouse hepatocytes for their clonal expansion to HCC.

In this context, it should be noted that the results of experimental protocol 4 are apparently in contrast with those of Ngo et al,35 who reported 100% of Nrf2 mutation in mouse HCCs induced by a single dose of DEN in the absence of promoting procedures. Although experimental protocol 4 closely resembles that used by Ngo et al,35 differences nevertheless exist that may be responsible for the discrepancy concerning the frequency of Nrf2 mutation, as follows: (1) dose of DEN (15 vs 25 mg/kg); (2) route of administration: gavage vs intraperitoneal administration; (3) strain: B6C3F1 vs C57BL6/129SV; and (4) sex: females vs males. Although some of these differences (ie, dose or route of administration) were unlikely to account for the lack of Nrf2 mutation observed in our work, the different strain used in the present work may be the key to explain the discrepancy between the 2 studies. Indeed, although the incidence of Ha-ras gene mutations in spontaneously developed liver tumors was 50%–63% in mice of the same strain used in our study (B6C3), DEN-induced tumors showed a very low incidence of Ha-ras mutations (1 of 13 tumors; 7.7%).43 On the other hand, the BraV637E mutation, corresponding to the human BraV600E mutation, was detected in 54 of 63 (85.7%) hepatic lesions induced by neonatal treatment with DEN in mice of the same strain used in our study (B6C3F1).44 Most important, the same work showed that whole-exome analysis performed in 4 tumors generated by neonatal treatment with DEN in B6C3F1 mice was able to identify 98 mutations, but none of them involved the Nrf2 gene. Thus, the different strains used in our study and the study by Ngo et al35 may well be responsible for the different results.

Independently of mutations, the present study did not show clear evidence of activation of the Keap1–Nrf2 pathway in the examined models. Indeed, the expression of Nrf2 target genes, such as Nqo1, Hmxo1, Gclc, and Gsta4 was not significantly changed in most of the tumors. The latter finding reasonably rules out the possibility that other mechanisms, such as increased nuclear translocation and transcriptional activity resulting from p62 accumulation, Nrf2 deglycation by fructosamine-3, Keap1 succination, and degradation by fumarate or other oncometabolite,45 could be involved in mouse HCC development.

As mentioned previously, Nrf2 redirects glucose and glutamine into anabolic pathways suggestive of the metabolic reprogramming often associated with cancer cells.13 According to the lack of Nrf2 mutations and its transcriptional activation, no significant and convincing evidence of metabolic reprogramming was found in the present study, as documented by the lack of increase of Glut1, G6pdx, and Mct4 (representative of increased glucose consumption, PPP activation, and lactate extrusion), or Gls, responsible for glutaminolysis. Using a DEN + phenobarbital mouse experimental model, Unterberger et al46 showed that both Ha-ras and Ctnnb1 mutated tumors showed a reduction in the levels of glucose-6-phosphatase, a condition favoring tumor cells because this enzyme catches glucose as an energy source. However, although glucose-6-phosphatase may be used through the PPP, it was up-regulated in Ha-ras, but not in Ctnnb1, mutated tumors. It also was surprising that although transcriptional up-regulation of Tricarboxylic Acid Cycle (TCA) cycle enzymes, such as isocitrate dehydrogenase and citrate synthase, was observed in Ctnnb1 mutated tumors, increased lactate levels were observed in Ha-ras but not in Ctnnb1 mutated tumors. These results were in line with those obtained by Yuneva et al,47 who did not detect increased lactate levels in MET-induced mouse liver tumors characterized by activating β-catenin mutations, and suggested that glucose and glutamine metabolism in HCC varies with the nature of the activated oncogene.

In conclusion, our present study, unlike rat HCCs, shows the following: (1) no Nrf2 mutation takes place in HCCs generated by distinct mouse models of chemically induced hepatocarcinogenesis (3 strains, both sexes, different ages of animals, and different doses of DEN); (2) no increased activation of the Keap1–Nrf2 pathway was observed in the same tumors; and (3) no change in the expression of genes involved in glycolysis, PPP, and glutamine pathway indicative of metabolic reprogramming was observed in mouse HCCs lacking Nrf2 activation but carrying Ctnnb1 mutations. These results also suggest that for translational studies investigating the role of Nrf2 mutation/activation in chemically induced hepatocarcinogenesis, the mouse may not be the ideal model because it does not recapitulate the human landscape.

Materials and Methods

Female C3H mice (ages, 6–8 wks) were obtained from Charles River (Milan, Italy). Two-week-old C57BL/6 male mice were obtained from Charles River, and maintained at the University Amedeo Avogadro of East Piedmont (Novara, Italy). Seven-day-old B6C3F1 female mice (Charles River) were kept at the Istituto Nazionale Tumori (Milan, Italy). Guidelines for the Care and Use of Laboratory Animals were followed throughout the investigation. All animal procedures were approved by the Ethical Commission of the University of Cagliari, East Piedmont, and the Italian Ministry of Health.

Experimental Protocol 1

Mice were injected intraperitoneally with DEN at a dose of 90 mg/kg body weight. After a 1-week recovery period, mice were treated intragastrically with TCPOBOP (3 mg/kg

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body weight; Sigma-Aldrich, Milan, Italy) once weekly for 28 weeks. Another group of mice received TCPOBOP alone, once a week for 42 weeks. Untreated mice were used as a further control group.

**Experimental Protocol 2**
Mice were treated with intragastric injections of TCPOBOP (3 mg/kg body weight) once weekly for 42 weeks. Age-matched mice treated with dimethyl sulfoxide dissolved in corn oil were used as controls.

**Experimental Protocol 3**
A single intraperitoneal injection of DEN (25 mg/kg body weight) was given to 2-week-old male pups (C57BL/6 mice). At 6 weeks of age, animals were fed a CDA obtained from Laboratorio Dottori Piccioni (Gessate, Italy) for the following 25 weeks.

**Experimental Protocol 4**
Seven-day-old B6C3F1 female mice received a single dose of DEN (15 mg/kg body weight in 0.9% NaCl solution) by gavage. Animals were killed 40 weeks later.

**DNA Sequencing**
Total RNA was extracted from frozen liver samples using TRIzol Reagent (Thermo Fisher Scientific), according to the manufacturer’s protocol. Total RNA was retrotranscribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Milan Italy). To identify Nrf2 mutations, we amplified exon 2 of the mouse Nrf2 gene, using a touch-down PCR protocol (annealing temperature, 66°C–60°C). To analyze mouse cDNA sequences corresponding to the second exon of Nrf2, we designed the following 2 primers: forward 5′-CTCTGCTGCAATGATCCCTC-3′ and reverse 5′-CAGGGCAAGGACTCATGG-3′. All PCR products were amplified with High-Fidelity Taq polymerase (Platinum Taq DNA Polymerase High Fidelity; Invitrogen), purified (by exonuclease 1 and shrimp alkaline phosphatase), and sequenced by fluorescent-based Sanger direct sequencing in an ABI3130 DNA capillary sequencer (Thermo Fisher Scientific, Milan, Italy).

**Microarray Analysis**
The microarray expression profiles of RNA in HCCs and adjacent noncancerous tissues described in our previous study29 were re-analyzed from Gene Expression Omnibus (accession number: GSE113708). Analysis was performed in R studio (https://cran.r-project.org/bin/windows/base/). Quantile normalized data were downloaded using Geoquery (https://www.bioconductor.org/packages/release/bioc/html/GEQ query.html) and the limma package (https://bio conductor.org/packages/release/bioc/html/limma.html) was applied to perform gene set testing and differential gene expression. Only genes whose expression differed by at least 1.5-fold were considered for further analysis.

**Functional Analysis Using IPA**
Rat standard gene symbols (RGD ids) were submitted to the Ingenuity IPA analysis pipeline. Analysis of the pathways was based on the number of genes significantly dysregulated (fold difference cut-off, ±1.5) with corresponding biological functions. The significance of each network and the connectivity was estimated in IPA.

**qRT-PCR Analysis**
The same cDNA used for gene sequencing also was used for qRT-PCR analysis. Total and microdissected RNA was retrotranscribed using the High-Capacity cDNA Reverse-Transcription Kit (Thermo Fisher Scientific). Analysis of Nqo1, Hmox1, Gclc, Gsta4, Glut1, Mct4, G6pdx, Hk2, G6pc, and Glu was performed using specific TaqMan probes (Thermo Fisher Scientific) and Gapdh as the reference gene.

**Immunohistochemistry**
Liver sections were fixed in 10% buffered formalin and processed for staining with H&E or immunohistochemistry. Paraffin-embedded tissue was cut into 4-μm sections, dewaxed, and hydrated. Endogenous peroxide was inactivated using hydrogen peroxide. Slides were microwaved in citrate buffer at pH 6.0 (ab93678; Abcam, Cambridge, UK) or in EDTA buffer at pH 8.0 (ab64216; Abcam), following an overnight incubation with the primary antibodies Nqo1 (ab28947; Abcam), G6pd (ab87230; Abcam), and Glu (ab262716; Abcam). After washes, the sections were incubated with the appropriate polymer DAKO Envision (Milan, Italy) secondary antibody at room temperature. Signal was detected using the VECTOR NovaRED Peroxidase (horse-radish peroxidase) Substrate Kit (Vector Laboratories, Burlingame, CA). Sections were counterstained with Harris hematoxylin solution (Sigma-Aldrich) and passed through the dehydration process and covered.

**Statistical Analysis**
Statistical significance was performed using the Student t test with Instat (GraphPad, San Diego, CA). The results of observations are presented as the means ± SD value. P < .05 was considered a significant difference between groups.
All authors had access to the study data and reviewed and approved the final manuscript.

**References**
1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer
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2. Lau A, Villeneuve NF, Sun Z, Wong PK, Zhang DD. Dual roles of Nrf2 in cancer. Pharmacol Res 2008; 58:262–270.

3. Sporn MB, Liby KT. Nrf2 and cancer: the good, the bad and the importance of context. Nat Rev Cancer 2012; 12:564–571.

4. Menegon S, Columbano A, Giordano S. The dual roles of Nrf2 in cancer. Trends Mol Med 2016;22:578–593.

5. Motohashi H, Yamamoto M. Nrf2-Keap1 defines a physiologically important stress response mechanism. Trends Mol Med 2004;10:549–557.

6. Kobayashi A, Kang M-I, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K, Yamamoto M. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol Cell Biol 2004;24:7130–7139.

7. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 1999;13:76–86.

8. Kobayashi A, Kang M-I, Watai Y, Tong KI, Shibata T, Uchida K, Yamamoto M. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. Mol Cell Biol 2006;26:221–229.

9. McMahon M, Itoh K, Yamamoto M, Hayes JD. Keap1-dependent proteoglycan degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. J Biol Chem 2003;278:21592–21600.

10. Takahashi T, Sonobe M, Menju T, Nakayama E, Mino N, Iwakiri S, Nagai S, Sato K, Miyahara R, Okubo K, Hirata T, Date H, Wada H. Mutations in Keap1 are a potential prognostic factor in resected non-small cell lung cancer. J Surg Oncol 2010;101:500–506.

11. Ohta T, Iijima K, Miyamoto M, Nakahara I, Tanaka H, Ohtsuji M, Suzuki T, Kobayashi A, Yokota J, Sakiyama T, Shibata T, Yamamoto M, Hirohashi S. Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. Cancer Res 2008;68:1303–1309.

12. Solis LM, Behrens C, Dong W, Suraokar M, Ozburn NC, Moran CA, Corvalan AH, Biswal S, Swisher SG, Bekele BN, Minna JD, Stewart DJ, Wistuba II. Nrf2 and Keap1 abnormalities in non-small cell lung carcinoma and association with clinicopathologic features. Clin Cancer Res 2010;16:3743–3753.

13. Nioi P, Nguyen T. A mutation of Keap1 found in breast cancer impairs its ability to repress Nrf2 activity. Biochem Biophys Res Commun 2007;362:816–821.

14. Yoo NJ, Kim HR, Kim YR, An CH, Lee SH. Somatic mutations of the Keap1 gene in common solid cancers. Histopathology 2012;60:943–952.

15. Konstantinopoulos PA, Spentzos D, Fountzilas E, Francoeur N, Sanisetty S, Grammatikos AP, Hecht JL, Cannistra SA. Keap1 mutations and Nrf2 pathway activation in epithelial ovarian cancer. Cancer Res 2011; 71:5081–5089.

16. Guichard C, Amaddeo G, Imbeaud S, Ladeiro Y, Pelletier L, Maad I Ben, Calderaro J, Bioulac-Sage P, Letexier M, Degos F, Clément B, Balabaud C, Chevet E, Laurent A, Couchy G, Lotouézé E, Calvo F, Zucman-Rossi J. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet 2012; 44:694–698.

17. Cleary SP, Jeck WR, Zhao X, Chen K, Selitsky SR, Savich GL, Tan T-X, Wu MC, Getz G, Lawrence MS, Parker JS, Li J, Powers S, Kim H, Fischer S, Quind M, Ghanevar A, Chang DY. Identification of driver genes in hepatocellular carcinoma by exome sequencing. Hepatology 2013;58:1693–1702.

18. Sanghvi VR, Leibold J, Mina M, Mohan P, Berishaj M, Li Z, Miele MM, Lailler N, Zhao C, de Stanchina E, Viale A, Akkari L, Lowe SW, Ciriello G, Hendrickson RC, Wendel H-G. The oncogenic action of Nrf2 depends on de-glucylation by fructoseamine-3-kinase. cell 2019; 178:807–819.e21.

19. Zhang M, Zhang C, Zhang L, Yang Q, Zhou S, Wen Q, Wang J. Nrf2 is a potential prognostic marker and promotes proliferation and invasion in human hepatocellular carcinoma. BMC Cancer 2015;15:531.

20. Zavattari P, Perra A, Menegon S, Kowalik MA, Petralli A, Angioni MM, Follenzi A, Quagliata L, Ledda-Columbano GM, Terracciano L, Giordano S, Columbano A. Nrf2, but not β-catenin, mutation represents an early event in rat hepatocarcinogenesis. Hepatology 2015;62:851–862.

21. Onami C, Szydloowska M, Taguchi K, Zavattari P, Perra A, Yamamoto M, Columbano A. Genetic inactivation of Nrf2 prevents clonal expansion of initiated cells in a nutritional model of rat hepatocarcinogenesis. J Hepatol 2018; 69:635–643.

22. Onami C, Perra A, Kowalik MA, Rizzolio S, Puliga E, Cabras L, Giordano S, Columbano A. Distinct mechanisms are responsible for Nrf2-Keap1 pathway activation at different stages of rat hepatocarcinogenesis. Cancers (Basel) 2020;12:2305.

23. Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, Sou Y-S, Ueno I, Sakamoto A, Tong KI, Kim M, Nishito Y, Iemura S, Natsume T, Ueno T, Komina E, Motohashi H, Tanaka K, Yamamoto M. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. Nat Cell Biol 2010;12:213–223.

24. Umemura A, He F, Taniguchi K, Nagakawa H, Yamachika S, Font-Burgada J, Zhong Z, Subramaniam S, Raghunandan S, Duran A, Linares JF, Reina-Campos M, Umemura S, Valasek MA, Seki E, Yamaguchi K, Koike K, Itoh Y, Diaz-Meco MT, Moscat J, Karin M. p62, Upregulated during preneoplasia, induces hepatocellular carcinogenesis by maintaining survival of stressed HCC-initiating cells. Cancer Cell 2016; 29:935–948.
associated with NF-E2-related factor 2 activation in hepatocellular carcinoma. J Hepatobiliary Pancreat Sci 2016;23:467–471.

26. Yasuda D, Ohe T, Takahashi K, Imamura R, Kojima H, Okabe T, Ichimura Y, Komatsu M, Yamamoto M, Nagano T, Mashito T. Inhibitors of the protein-protein interaction between phosphorylated p62 and Keap1 attenuate chemoresistance in a human hepatocellular carcinoma cell line. Free Radi Res 2020;54:859–871.

27. Savall M, Senni N, Lagoutte I, Sohier P, Dentin R, Romagnolo B, Peret C, Bossard P. Cooperation between the Nrf2 pathway and oncogenic β-catenin during HCC tumorigenesis. Hepatol Commun 2021;5:1490–1506.

28. Tao J, Krutsenko Y, Moghe A, Singh S, Poddar M, Bell A, Oertel M, Singh AD, Geller D, Chen X, Lujambio A, Liu S, Monga SP. Nrf2 and β-catenin coactivation in hepatocellular cancer: biological and therapeutic implications. Hepatology 2021;74:741–759.

29. Mattu S, Saliba C, Sulas P, Zavattari P, Perra A, Kowalik MA, Monga SP, Columbano A. High frequency of β-catenin mutations in mouse hepatocellular carcinomas induced by a nongenotoxic constitutive androstane receptor agonist. Am J Pathol 2018;188:2497–2507.

30. Rooney JP, Oshida K, Kumar R, Baldwin WS, Corton JC. Chemical activation of the constitutive androstane receptor leads to activation of oxidant-induced Nrf2. Toxicol Sci 2019;167:172–189.

31. Shibata T, Ohta T, Tong KL, Kokubu A, Odogawa R, Tsuta K, Asamura H, Yamamoto M, Hirohashi S. Cancer related mutations in Nrf2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. Proc Natl Acad Sci U S A 2008;105:13568–13573.

32. Petrelli A, Perra A, Cora D, Sulas P, Menegon S, Manca C, Migliore C, Kowalik MA, Ledda-Columbano GM, Giordano S, Columbano A. MicroRNA gene profiling unveiling early molecular changes and nuclear factor erythroid related factor 2 (Nrf2) activation in a rat model recapitulating human hepatocellular carcinoma (HCC). Hepatology 2014;59:228–241.

33. Mitsuishi Y, Taguchi K, Kawatani Y, Shibata T, Nukiwa T, Aburatani H, Yamamoto M, Motohashi H. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. Cancer Cell 2012;22:66–79.

34. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–674.

35. Ngo HKC, Kim D-H, Cha Y-N, Na H-K, Suh Y-J. Nrf2 mutagenic activation drives hepatocarcinogenesis. Cancer Res 2017;77:4797–4808.

36. Rojo de la Vega M, Chapman E, Zhang DD. Nrf2 and the hallmarks of cancer. Cancer Cell 2018;34:21–43.

37. Raghunath A, Sundarraj K, Arfuso F, Sethi G, Perumal E. Dysregulation of Nrf2 in hepatocellular carcinoma: role in cancer progression and chemoresistance. Cancers (Basel) 2018;10:481.

38. Heindryckx F, Colle I, Van Vlierberghe H. Experimental mouse models for hepatocellular carcinoma research. Int J Exp Pathol 2009;90:367–386.

39. De Minicis S, Kisseleva T, Francis H, Baroni GS, Benedetti A, Brenner D, Alvaro D, Alpini G, Marzioni M. Liver carcinogenesis: rodent models of hepatocarcinoma and cholangiocarcinoma. Dig Liver Dis 2013;45:450–459.

40. Jaworski M, Buchmann A, Bauer P, Riess O, Schwarz M. B-Raf and Ha-ras mutations in chemically induced mouse liver tumors. Oncogene 2005;24:1290–1295.

41. Buchmann A, Karcier Z, Schmid B, Strathmann J, Schwarz M. Differential selection for B-raf and Ha-ras mutated liver tumors in mice with high and low susceptibility to hepatocarcinogenesis. Mutat Res 2008;638:66–74.

42. Aydinlik H, Nguyen TD, Moennikes O, Buchmann A, Schwarz M. Selective pressure during tumor promotion by phenobarbital leads to clonal outgrowth of beta-catenin-mutated mouse liver tumors. Oncogene 2001;20:7812–7816.

43. Dragani TA, Manenti G, Colombo BM, Falvalva FS, Gariboldi M, Pierotti MA, Della Porta G. Incidence of mutations at codon 61 of the Ha-ras gene in liver tumors of mice genetically susceptible and resistant to hepatocarcinogenesis. Oncogene 1991;6:333–338.

44. Yamamoto M, Tanaka H, Xin B, Nishikawa Y, Yamazaki K, Shimizu K, Ogawa K. Role of the BrafV637E mutation in hepatocarcinogenesis induced by treatment with diethyltriamine in neonatal B6C3F1 mice. Mol Carcinog 2017;56:478–488.

45. Taguchi K, Yamamoto M. The KEAP1-NRF2 system as a molecular target of cancer treatment. Cancers (Basel) 2020;13:46.

46. Unterberger EB, Eichner J, Wzodeck C, Lempiäinen H, Luisier R, Terranova R, Metzger U, Plummer S, Knorpp T, Braeuning A, Mogs J, Templin MF, Honndorf V, Piotto M, Zell A, Schwarz M. Ha-ras and β-catenin oncoproteins orchestrate metabolic programs in mouse liver tumors. Int J Cancer 2014;135:1574–1585.

47. Yuneva MO, Fan TWM, Allen TD, Higashi RM, Ferraris DV, Tsukamoto T, Matés JM, Alonso FJ, Wang C, Seo Y, Chen X, Bishop JM. The metabolic profile of tumors depends on both the responsible genetic lesion and tissue type. Cell Metab 2012;15:157–170.

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