Published in final edited form as:

Hepatology. 2020 February ; 71(2): 549–568. doi:10.1002/hep.30818.

Nqo1 ablation inhibits activation of the PI3K/Akt and MAPK/ERK pathways and blocks metabolic adaptation in hepatocellular carcinoma

Manali Dimri¹, Ashley Humphries¹, Archana Laknaur¹, Sawsan Elattar¹, Tae Jin Lee², Ashok Sharma², Ravindra Kolhe³, Ande Satyanarayana¹

¹Department of Biochemistry and Molecular Biology, Molecular Oncology & Biomarkers Program, Georgia Cancer Center, Augusta University, Room-CN3150, 1410 Laney Walker Blvd., Augusta, GA 30912

²Center for Biotechnology and Genomic Medicine, Department of Population Health Sciences, Augusta University, GA, 30912

³Department of Pathology, Section of Anatomic Pathology, Augusta University, Augusta, GA 30912

Abstract

Cancer cells undergo metabolic adaptation to sustain uncontrolled proliferation. Aerobic glycolysis and glutaminolysis are two of the most essential characteristics of cancer metabolic reprogramming. Hyper-activated PI3K/Akt and MAPK/ERK signaling pathways play central roles in cancer cell metabolic adaptation since their downstream effectors, such as Akt and c-Myc, control most of the glycolytic and glutaminolysis genes. Here, we report that the cytosolic flavoprotein Nqo1 is strongly overexpressed in mouse and human hepatocellular carcinoma (HCC). Knockdown of *Nqo1* enhanced activity of the serine/threonine phosphatase PP2A, which operates at the intersection of the PI3K/Akt and MAPK/ERK pathways and dephosphorylates and inactivates PDK1, Akt, Raf, MEK and ERK1/2. *Nqo1* ablation also induced the expression of PTEN, a dual protein/lipid phosphatase that blocks PI3K/Akt signaling, via the ERK/CREB/c-Jun pathway. Together, Nqo1 ablation triggered simultaneous inhibition of the PI3K/Akt and MAPK/ERK pathways, suppressed the expression of glycolysis and glutaminolysis genes and blocked metabolic adaptation in liver cancer cells. Conversely, *Nqo1* overexpression caused hyper-activation of the PI3K/Akt and MAPK/ERK pathways and promoted metabolic adaptation. In **conclusion**, Nqo1 functions as an upstream activator of both the PI3K/Akt and MAPK/ERK pathways in liver cancer cells, and *Nqo1* ablation blocked metabolic adaptation and inhibited liver cancer cell proliferation and HCC growth in mice. Therefore, our results suggest that Nqo1 may function as a therapeutic target to inhibit liver cancer cell proliferation and inhibit HCC.

Correspondence Ande Satyanarayana, PhD, 1410 Laney Walker Blvd., Room-CN3150, Augusta, GA 30912, Phone: 706-723-0029, Fax: 706-721-0101, sande@augusta.edu.

Additional experimental procedures are provided in Suppl. Information.

The authors declare no conflicts of interest.

Keywords

HCC; PP2A; PTEN; Metabolic reprogramming; c-Myc; Aerobic glycolysis; Glutaminolysis

Introduction

The most fundamental feature of cancer cells is their ability to sustain uncontrolled proliferation via disruption of proliferative signaling pathways and loss of cell cycle regulation (1). Due to nonstop proliferation, cancer cells face a substantial bio-energetic challenge to produce sufficient energy and synthesize various biomolecules concurrently. Inevitably, this forces cancer cells to readapt their energy metabolism and undergo metabolic reprogramming, one of the central hallmarks of cancer (1, 2). A large number of cancer cells such as breast, colon, liver, lung, pancreas, prostate, thyroid, and bladder undergo metabolic adaptation (2, 3). The most well-known example of metabolic adaptation in cancer cells is the alteration of glucose metabolism. Irrespective of oxygen status, many cancer cells depend on high rates of glucose uptake and convert most of the glucose into lactate instead of completely catabolizing glucose by oxidative phosphorylation (OXPHOS), a phenomenon known as the Warburg effect (2, 3). Due to high glycolytic activity, the expression and activity of numerous glycolytic enzymes such as HK1, PFK1, enolase, LDH and PDK1 are highly elevated in cancer cells (2–4). In addition to aerobic glycolysis, glutaminolysis is also an essential characteristic of cancer metabolic reprogramming (5). Glutamine enters cells through SLC38A5 and SLC1A5 transporters and is converted to glutamate by glutaminase (GLS). Glutamate is converted to alpha-ketoglutarate (α -KG) by glutamate dehydrogenase, which enters the TCA cycle. Overall, cancer cells need to efficiently coordinate glycolysis and glutaminolysis to satisfy both bioenergetic and biosynthetic requirements for their proliferation and survival (2–4).

The signal transduction pathways that regulate aerobic glycolysis and glutaminolysis in cancer cells have been under extensive investigation. Accumulating evidence demonstrates that metabolic adaptation of cancer cells is a primary function of dysregulated, hyper-activated proliferative signaling pathways and loss of tumor suppressor functions (2, 4). Growth factors usually stimulate two key signal-transducing kinase pathways: the Ras/Raf/MAP kinase (MAPK/ERK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway. Most cancers harbor activating mutations of the master regulators (Ras, Raf, the p110 α PI3K subunit, and RTKs) or their downstream effectors (Akt, PDK1 and c-Myc) of the PI3K/Akt and MAPK/ERK pathways. The constitutive hyper-activation of the PI3K/Akt and MAPK/ERK signaling pathways can cause cancer-associated metabolic reprogramming (1, 6, 7). For example, activated PI3K/Akt leads to increased glucose transporter expression, activation of hexokinase and PFK1 leading to enhanced glucose uptake and glycolysis (8). On the other hand, c-Myc is one of the most frequently overexpressed genes in human cancers, and it promotes metabolic adaptation in cancer cells (9, 10). c-Myc regulates an array of genes that participate in both glucose and glutamine transport and metabolism, such as HK2, PFK, ENO1, GAPDH, PGK1, LDHA, PDK1, Glut1, glutaminase and the glutamine transporters, thereby exerting tremendous influence on cancer cell metabolic adaptation (9–12). Tumor suppressors, such as p53 and PTEN, negatively regulate activation of the

PI3K/Akt and MAPK/ERK pathways and suppress metabolic adaptation (6, 13, 14). However, they are often inactivated or lost in most cancers, making them unable to block metabolic reprogramming. Therefore, identification of factors that operate upstream of the PI3K/Akt and MAPK/ERK pathways could function as potential molecular targets to block cancer cell metabolic adaptation and inhibit tumor growth.

NAD(P)H Quinone Dehydrogenase 1 (Nqo1) is a cytosolic flavoprotein, and its expression is strongly induced in various cancers such as breast, pancreas, liver, bladder, ovary, thyroid, colorectal, cholangiocarcinoma, uterine cervical cancer, melanoma, and lung cancer (15–19). Increased expression of Nqo1 protein was also correlated with tumor size, advanced clinical stage and decreased patient survival rates in breast, lung and liver cancers (17, 19, 20), suggesting that Nqo1 could function as an oncogene. Nevertheless, how Nqo1 exerts its oncogenic effects, and through which pro-tumorigenic signaling pathways Nqo1 controls cancer cell proliferation, is not fully understood. Here, we have unraveled a novel function of Nqo1 in the regulation of the PI3K/Akt and MAPK/ERK pathways and metabolic adaptation in liver cancer cells.

Experimental procedures

Mice

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Augusta University, Georgia. Mice were bred and maintained under specific pathogen-free conditions with a 12 h light-dark cycle. *Nqo1*^{+/-} mouse frozen sperm were obtained from Dr. Minh Shong, Korea Research Institute of Bioscience and Biotechnology, South Korea. Using the Sperm Cryorecovery services of the Jackson Laboratory (project # 109311), we derived *Nqo1*^{+/-} mice. *Nqo1*^{-/-} and *Nqo1*^{+/+} litter-mate controls were generated by crossing *Nqo1*^{+/-} mice. A single intra-peritoneal injection of DEN to 15-day-old mice at a dose of 25mg/Kg body weight was given to induce liver tumors in male mice.

Human HCC samples

To analyze the expression of Nqo1 in human normal liver and in liver tumor tissues, 25 formalin-fixed, paraffin-embedded cases of liver cancer (American Joint Committee on Cancer stages I–IV) and 15 normal control liver samples were obtained from the pathology archives of Augusta University. The study protocol was reviewed and approved by the Institutional Review Board.

In vivo xenograft assay

Huh7 control and *Nqo1-KD* cells (6×10^6) were injected subcutaneously in the flank region of athymic nude mice (strain code # 490, Charles River Laboratories), and tumor growth was monitored daily. Mice were sacrificed after one month. Tumor weight and diameter were measured at the time of harvesting. A digital Vernier caliper was used to measure the tumor diameter.

Statistical analysis

All experiments were performed in triplicate. Statistical analyses were performed by 2-tailed Student's *t* test, and a value of $p < 0.05$ was considered statistically significant.

Results

Nqo1 is strongly upregulated in HCC

To discover novel genes that are involved in liver tumorigenesis, we performed RNA-Sequencing (*RNA-Seq*) by comparing non-tumorous normal livers versus diethylnitrosamine (DEN) chemical-induced liver tumors in mice. After applying a fold-change of 4 ($FC > 4$) and $FDR < 0.001$ (false discovery rate) and ranking the genes based on significance (p value), we identified 318 downregulated and 648 upregulated genes in liver tumors compared to non-tumorous tissues (Fig 1A, Suppl. Tables 1 & 2). Out of 648 upregulated genes, NAD(P)H Quinone Dehydrogenase 1 (*Nqo1*) ranked at 138 with a 14.64 fold upregulation (Fig 1A–C, Suppl. Table 1). We selected *Nqo1* for further investigation since it fulfilled all the other criteria we have set forth such as: **A)** The Human Protein Atlas database showed strong *Nqo1* expression in ~40–50% of human liver tumors and no expression in normal livers. This information is either not available for several genes that ranked above *Nqo1* or they are not strongly expressed. **B)** Re-confirmation of *RNA-Seq* data by Western blotting revealed strong expression of *Nqo1* in mouse liver tumors (Fig 1D). **C)** Liver cancer cell lines HepG2 and Huh-7 showed strong expression of *Nqo1* where as its expressed is very low in H2.35 primary hepatocyte cell line (Fig 1E). **D)** An extensive literature search revealed no previous studies showing the specific function of *Nqo1* in HCC. **E)** Increased expression of *Nqo1* protein was correlated with tumor size, advanced clinical stage and decreased patient survival rate in liver cancers (20). Genes that ranked above *Nqo1* did not meet one or more of the above criteria, whereas *Nqo1* fulfilled all the criteria providing a strong rationale for thoroughly investigating its function in HCC.

To determine if strong expression of *Nqo1* in mouse liver tumors translate into the human condition, we analyzed *Nqo1* expression in human HCC samples. Non-tumorous liver tissues (15 samples) did not show any significant *Nqo1* staining (Fig 1F). Out of 25 HCC samples stained, we detected strong and predominantly cytosolic expression of *Nqo1* in 14 (~60%) HCC samples (Fig 1G–J). All the 14 samples that stained positive for *Nqo1* predominantly showed strong (IHC score 2) to very strong (IHC score 3) staining intensity (Fig 1K). Additionally, we also performed IHC on DEN-induced mouse HCC samples and again detected predominantly strong cytosolic staining in all 6 mouse HCC samples, whereas non-tumorous tissues showed weak *Nqo1* staining in the sinusoidal cells (Fig 1L–M). These observations suggest that *Nqo1* might play a role in HCC initiation and/or progression.

Nqo1 knockdown (*Nqo1-KD*) suppressed liver cancer cell proliferation, colony formation and xenograft growth

Strong expression of *Nqo1* in liver tumors and in liver cancer cell lines raises the question if *Nqo1* is required for the proliferation, survival and/or migration of liver cancer cells. Hence, we knocked down *Nqo1* in Huh-7 cells using *shRNA* (Fig 2A). *Nqo1-KD* cells proliferated

at a significantly slower rate compared to control cells (Fig 2B), and we did not detect any significant cell death in *Nqo1-KD* cell cultures. *Nqo1-KD* cells also displayed a decreased migration rate (Fig 2C–D). Moreover, *in vitro* colony formation ability of *Nqo1-KD* cells was also significantly impaired (Fig 2E–F). To further investigate if *Nqo1* is required for tumor growth *in vivo*, we performed subcutaneous xenografts in athymic nude mice, which revealed a significant reduction in xenograft tumor growth of *Nqo1-KD* cells compared to control cells (Fig 2G–J). These observations suggest that *Nqo1* is required for liver cancer cell proliferation and tumor growth *in vivo*.

DEN-induced HCC is strongly inhibited in *Nqo1* knockout (*Nqo1*^{−/−}) mice

To study the impact of loss of *Nqo1* on HCC growth in a more physiologically relevant system, we utilized *Nqo1*^{+/+} control and *Nqo1*^{−/−} mice. *Nqo1*^{−/−} mice are normal and fertile, and did not show any significant phenotypes. We performed the well-established DEN-induced HCC in *Nqo1*^{+/+} and *Nqo1*^{−/−} male mice. We detected a significantly reduced number of liver tumors in *Nqo1*^{−/−} compared to *Nqo1*^{+/+} mice 8 months after DEN treatment (Fig 3A). Tumor incidence as well as tumor size were significantly reduced in *Nqo1*^{−/−} compared to *Nqo1*^{+/+} mice (Fig 3B–D). Consistent with reduced liver tumor growth, the liver/body weight ratio was not increased in *Nqo1*^{−/−} mice (Fig 3E). These results demonstrate that absence of *Nqo1* significantly inhibited HCC growth. Histological analysis of liver tumors revealed large tumor cells with hyperchromatic nuclei in a compact growth pattern in *Nqo1*^{+/+} mice, whereas *Nqo1*^{−/−} livers appeared normal (Fig 3F). We also detected reduced lipid accumulation (Fig 3G) and fibrosis (Fig 3H) in *Nqo1*^{−/−} compared to *Nqo1*^{+/+} mouse livers. *Nqo1*^{−/−} liver tumors also displayed significantly reduced proliferation as revealed by PCNA staining (Fig 3I), indicating that reduced tumor growth in *Nqo1*^{−/−} mice was due to decreased proliferation. Overall, these results indicate that absence of *Nqo1* inhibits liver tumor growth.

Nqo1-KD resulted in strong upregulation of PTEN, impaired activation of Akt and ERK1/2 and downregulation of c-Myc

Our gene expression analysis revealed 648 upregulated and 318 downregulated genes in liver tumors compared to normal livers (Fig 1A, Suppl. Table 1–2). Transcriptome Analysis Console (TAC) pathway mapping on these 966 differentially regulated genes revealed that 77 differentially expressed genes belong to the PI3K/Akt/mTOR and MAPK/ERK signaling pathways (Suppl. Fig 3). These two signaling pathways ranked in the top 5 dysregulated pathways in liver tumors based on the total number of differentially expressed genes in each pathway (Suppl. Fig 3). Since *Nqo1* is significantly overexpressed (14.64 fold) in liver tumors and its knockdown strongly inhibited liver cancer cell proliferation and tumor growth (Fig 2–3), we wondered if *Nqo1* induces its oncogenic effects via some of the major pro-oncogenic signaling pathways such as the PI3K/Akt/mTOR and MAPK/ERK pathways. To investigate this possibility, we analyzed the expression and/or activity status of some of the critical components of these two pathways in control and *Nqo1-KD* Huh-7 cells. Very fascinatingly, we detected strong upregulation of PTEN and impaired phosphorylation and activation of PDK1 and Akt, whereas mTOR levels were not significantly altered in response to *Nqo1-KD* (Fig 4A). These observations suggest that *Nqo1* is required for activation of the PI3K/Akt pathway. PTEN is a dual protein/lipid phosphatase that blocks PI3K/Akt signaling

by dephosphorylating phosphatidylinositol,3,4,5 triphosphate (PIP3) to PIP2 (1, 6, 7, 13). To evaluate if *Nqo1-KD*-associated PTEN upregulation affected PIP2/PIP3 levels, we measured and detected significantly elevated cellular PIP2 levels in *Nqo1-KD* cells compared to control cells (Fig 4B), further indicating that *Nqo1-KD* upregulates PTEN and blocks activation of the PI3K/Akt pathway.

Next, to investigate if Nqo1 also regulates the MAPK/ERK pathway, we analyzed the activity status of some of the main components of this pathway. Very interestingly, *Nqo1-KD* resulted in a significant reduction in the phosphorylation and activation of MEK1 and ERK1/2 and reduced inhibitory phosphorylation at Ser9 of GSK3 β (active form) (Fig 4C), suggesting that Nqo1 is required for stimulation of the MAPK/ERK pathway, and in the absence of Nqo1, MAPK/ERK activation is blunted. Both the PI3K/Akt and MAPK/ERK pathways regulate c-Myc expression and stability (21). Since *Nqo1-KD* blunted the activation of both of these pathways, consequently, we detected significant downregulation of c-Myc (Fig 4A). To determine if these *in vitro* observations translate into *in vivo*, we analyzed the expression and/or activity status of the components of the PI3K/Akt and MAPK/ERK pathways in *Nqo1^{+/+}* and *Nqo1^{-/-}* mouse liver tumors. Again, we detected upregulation of PTEN, and impaired phosphorylation and activation of PDK1, Akt, MEK1 and ERK1/2, and downregulation of c-Myc in *Nqo1^{-/-}* compared to *Nqo1^{+/+}* liver tumors (Fig 4D). Overall, our observations suggest that Nqo1 is required for the activation of both the PI3K/Akt and MAPK/ERK signaling pathways, and in the absence of Nqo1, activation of these pathways are blunted.

***Nqo1-KD* enhanced the activity of PP2A, which is a negative regulator of the PI3K/Akt and MAPK/ERK pathways**

There is a substantial amount of cross-regulation between the PI3K/Akt and MAPK/ERK signaling pathways, and at several critical regulatory points these pathways also intertwine with other signaling pathways (22, 23). Some of the factors that are involved in the cross-regulation of the PI3K/Akt and MAPK/ERK pathways are PTEN, P53, AMPK, GSK3 β and PP2A (22, 23). To evaluate if Nqo1 regulates any of these factors, we analyzed their expression which revealed enhanced expression of PTEN in *Nqo1-KD* cells (Fig 4A and D). PTEN is not only a negative regulator of the PI3K/Akt pathway, but also inhibits the MAPK/ERK pathway in certain cell types (22, 24). We did not detect any significant difference in the expression of p53, AMPK and pAMPK (Fig 4E). We then examined if Nqo1 also influences the expression and/or activity of the serine/threonine phosphatase PP2A, one of the strongest molecular links between the PI3K/Akt and MAPK/ERK pathways (25–28).

PP2A comprises a scaffolding subunit (A), a catalytic subunit (C) and a regulatory subunit (B) that provides functional specificity to the PP2A hetero-trimer. PP2A operates at the intersection of the PI3K/Akt and MAPK/ERK pathways, and dephosphorylates and inactivates PDK1, Akt, Raf, MEK and ERK1/2, thereby shutting down these pathways simultaneously (28, 29). Analysis of PP2A subunit levels revealed no difference between control and *Nqo1-KD* cells (Fig 4E). Hence, we deliberated whether *Nqo1-KD* affected PP2A activity directly or indirectly, which in turn caused reduced activation of MEK, ERK

and Akt. Analysis of PP2A enzymatic activity revealed significantly enhanced PP2A phosphatase activity in *Nqo1-KD* cells (Fig 4F), suggesting that Nqo1 interferes with PP2A activity, and absence of Nqo1 causes enhanced PP2A activity, leading to reduced phosphorylation of MEK, ERK and Akt. To further validate that enhanced PP2A activity in *Nqo1-KD* cells was responsible for reduced phosphorylation of the kinases in the PI3K/Akt and MAPK/ERK pathways, we treated *Nqo1-KD* cells with different concentrations of the PP2A inhibitor LB-100 for 24 h (Suppl. Fig 5A). The phospho PDK1, Akt, MEK1 and ERK1/2 levels were increased in *Nqo1-KD* cells in response to LB-100 treatment in a concentration-dependent manner (Suppl. Fig 5B). Since we detected higher PP2A activity in *Nqo1-KD* cells compared to control cells (Fig 4F), we asked if we reduce PP2A activity in *Nqo1-KD* cells to control cell levels will it rescue phosphorylation of kinases in the PI3K/Akt and MAPK/ERK pathways to control cell levels. Thus, we treated *Nqo1-KD* cells with LB-100 (10 μ M) for 48 h, which reduced PP2A activity in *Nqo1-KD* cells to approximately equal to control cell levels (Fig 4G). Phospho PDK1, Akt, MEK1/2 and ERK1/2 in LB-100 treated *Nqo1-KD* cells was significantly rescued and almost comparable to control cells (Fig 4H). These findings suggest that increased activity of PP2A in *Nqo1-KD* cells contributed to decreased phosphorylation and activation of kinases in the PI3K/Akt and MAPK/ERK pathways.

***Nqo1-KD* enhanced *Pten* transcription**

PTEN is upregulated in *Nqo1-KD* Huh-7 cells and in *Nqo1*^{-/-} mouse liver tumors (Fig 4A and 4D), suggesting that Nqo1 regulates activation of the PI3K/Akt pathway by controlling PTEN expression. Next, we asked at what level Nqo1 regulates PTEN. Using qPCR, we detected a significant increase in *Pten* mRNA levels in *Nqo1-KD* cells (Fig 5A), suggesting that Nqo1 regulates PTEN at the transcriptional level. Since Nqo1 is not a transcription factor, we postulated that Nqo1 may regulate *Pten* expression via other factors that either induce or suppress *Pten* transcription. EGFR, PPAR γ and p53 induce *Pten* expression, whereas TGF β , NF κ B, Hes1 and c-Jun bind to the *Pten* promoter and suppress its expression (30, 31). Analysis of expression levels of some of these factors revealed a significant reduction in c-Jun expression in *Nqo1-KD* cells (Fig 5B).

How does Nqo1 regulate *Pten* transcription via c-Jun? The ERK/CREB/c-Jun pathway is known to inhibit *Pten* transcription. ERK1/2 phosphorylates and activates CREB, which can induce *c-Jun* expression. c-Jun directly binds to the *Pten* promoter and inhibits its transcription (30–32). Thus, we postulated that Nqo1 maintains ERK1/2 in its active phospho form by inhibiting PP2A, which phosphorylates and activates CREB. Activated CREB induces *c-Jun* expression, which binds to the *Pten* promoter and inhibits its expression (Fig 5C). In the absence of Nqo1, active PP2A dephosphorylates and inactivates ERK1/2, leading to blunted CREB activation, diminished expression and reduced binding of c-Jun to the *Pten* promoter, causing elevated expression of *Pten*. Consistent with this idea, we detected reduced levels of pCREB and c-Jun and elevated expression of PTEN in *Nqo1-KD* cells (Fig 5D). To evaluate if PP2A-mediated inactivation of CREB is responsible for downregulation of c-Jun and elevated expression of PTEN, we inhibited PP2A activity in *Nqo1-KD* cells by LB-100, which resulted in increased pCREB and c-Jun levels and reduced expression of PTEN (Fig 5E). In accordance with the reduced levels of c-Jun in

Nqo1-KD cells, by CHIP-qPCR, we detected reduced c-Jun binding to the *Pten* promoter in *Nqo1-KD* cells (Fig 5F). To further evaluate if the absence of Nqo1 terminated the ERK/CREB/c-Jun pathway, leading to enhanced expression of *Pten*, we overexpressed the constitutively active form of *Creb* (VP16-CREB) in *Nqo1-KD* cells, which caused c-Jun induction and PTEN suppression (Fig 5G), suggesting that Nqo1 controls *Pten* expression via the ERK/CREB/c-Jun pathway. Similar to our *in vitro* observations, we also detected increased expression of *Pten* mRNA (Fig 5H), and reduced expression of pCREB and c-Jun and increased expression of PTEN in *Nqo1^{-/-}* compared to *Nqo1^{+/+}* liver tumors (Fig 5I). Moreover, IHC also revealed more intense PTEN staining in *Nqo1^{-/-}* compared to *Nqo1^{+/+}* liver tumors (Fig 5J). We detected increased *Pten* mRNA and protein levels not only in the liver tumors but also in normal livers of *Nqo1^{-/-}* compared to *Nqo1^{+/+}* mice (Fig 5K–L). Overall, these observations suggest that PTEN is one of the main downstream targets of Nqo1.

***Nqo1-KD* suppressed aerobic glycolysis**

The downstream effectors of the PI3K/Akt and MAPK/ERK pathways play a central role in cancer cell metabolic reprogramming (1, 6, 7). For example, hyperactivation of Akt promotes aerobic glycolysis and glutaminolysis (4, 33), and cMyc activates the transcription of various glycolytic and glutaminolysis genes (9–11). Since *Nqo1* ablation caused significant impairment in the activation of Akt and expression of c-Myc (Fig 4A and D), we wondered whether Nqo1 controls metabolic adaptation in cancer cells, which would explain impaired proliferation of liver cancer cells and HCC growth in response to *Nqo1* ablation (Fig 2–3). To investigate this possibility, we first measured glucose uptake and lactate production rates between control and *Nqo1-KD* Huh-7 cells, which revealed reduced glucose uptake in *Nqo1-KD* cells (Fig 6A). Consequently, lactate production was significantly reduced in *Nqo1-KD* cells compared to control cells (Fig 6B). These observations encouraged us to speculate whether *Nqo1-KD* caused the cells to switch from aerobic glycolysis to OXPHOS. To this end, we measured oxygen consumption rates (OCRs) and detected significantly increased basal OCR and maximal respiratory capacity in *Nqo1-KD* cells compared to control cells (Fig 6C). *Nqo1-KD* cells also displayed reduced extracellular acidification rate (ECAR) (Fig 6D), an index of lactate production. These observations suggest that *Nqo1-KD* suppresses aerobic glycolysis and promotes OXPHOS in liver cancer cells.

To investigate if increased O₂ consumption in *Nqo1-KD* cells is due to an increase in mitochondrial content, we measured and observed no clear differences in mitochondrial mass and expression levels of the mitochondrial marker protein Cyt C between control and *Nqo1-KD* cells (Fig 6E, H), suggesting that *Nqo1-KD* could increase OXPHOS without affecting total mitochondrial content. To determine if glycolytic activity was indeed reduced in response to *Nqo1-KD*, we analyzed the relative expression levels of glycolytic genes by qPCR and detected significant downregulation of HK2, Glut1, PKM2, aldolase-A, enolase-1, enolase-2, LDHA and PDHK1 mRNA in *Nqo1-KD* cells (Fig 6F). We also detected significantly reduced expression of glutaminase and glutamine transporters SN1, SN2 and SLC1A5 at the mRNA level in *Nqo1-KD* cells compared to control cells (Fig 6F–G). Further analysis at the protein level also revealed downregulation of glycolytic enzymes

such as HK2, aldolase A, enolase-1, enolase-2 and glutaminase in *Nqo1-KD* cells (Fig 6H). Consistent with the reduced expression, HK2 enzymatic activity was significantly reduced in *Nqo1-KD* cells compared to control cells (Fig 6I). Not only *Nqo1-KD* Huh-7 cells but also *Nqo1^{-/-}* liver tumors displayed reduced expression of glycolytic enzymes and GLS (Fig 6J), and glucose and lactate levels were significantly less in *Nqo1^{-/-}* compared to *Nqo1^{+/+}* liver tumors (Fig 6K–L). Overall, these results suggest that *Nqo1* ablation caused reduced expression of glycolytic and glutaminolysis genes, leading to suppression of aerobic glycolysis and glutaminolysis.

***Nqo1* overexpression (*Nqo1-OE*) downregulated PTEN, activated Akt and ERK1/2, and induced c-Myc expression, leading to enhanced aerobic glycolysis**

To further validate if *Nqo1* is, in fact, promoting metabolic adaptation in liver cancer cells by activating the PI3K/Akt and MAPK/ERK pathways, we transiently overexpressed human *Nqo1* in Huh-7 cells. *Nqo1-OE* cell proliferation is increased (Fig 7A), and glucose uptake and lactate production are also increased in *Nqo1-OE* cells compared to control cells (Fig 7B–C). *Nqo1-OE* cells also displayed reduced OCR and increased ECAR compared to control cells (Fig 7D–E). These observations suggest that *Nqo1-OE* enhances aerobic glycolysis in liver cancer cells. To determine if increased aerobic glycolysis in response to *Nqo1-OE* is due to activation of the PI3K/Akt and MAPK/ERK pathways, we analyzed and detected significantly diminished expression of PTEN and increased expression of c-Jun (Fig 7F), which suppresses *Pten* expression. We also observed increased activation of ERK and Akt and induced expression of c-Myc and glycolytic genes in *Nqo1-OE* cells compared to control cells (Fig 7F). These results further confirm that *Nqo1* promotes metabolic adaptation in liver cancer cells by simultaneously activating the PI3K/Akt and MAPK/ERK pathways. Based on these observations, we questioned if enhanced expression of *Nqo1* is sufficient to stimulate the PI3K/Akt and MAPK/ERK pathways and drive metabolic adaptation even in the primary hepatocytes. We overexpressed *Nqo1* in H2.35 primary hepatocytes and observed induction of pCREB and c-Jun and downregulation of PTEN, and increased expression of pERK1/2, pAkt and c-Myc (Fig 7G). *Nqo1-OE* H2.35 cells also displayed elevated expression of glycolytic enzymes (Fig 7H) and increased glucose uptake and lactate production (Fig 7I–J). Also, *Nqo1-OE* cells displayed reduced OCR and increased ECAR compared to control cells (Fig 7K–L). These observations suggest that *Nqo1-OE* is sufficient to enhance aerobic glycolysis and drive metabolic adaptation in primary hepatocytes.

Akt and c-Myc overexpression partially rescued *Nqo1-KD*-associated phenotypes

Nqo1-KD caused impaired proliferation of liver cancer cells and downregulation of glycolysis and glutaminolysis genes (Fig 2 and 6). This is due to impaired activation of Akt and downregulation of c-Myc (Fig 4A, D), the downstream effectors of the PI3K/Akt and MAPK/ERK pathways. To determine if Akt and c-Myc are the direct and final downstream targets of *Nqo1*, we overexpressed constitutively active forms of *Akt* (Akt-T308D/S473D), and *c-Myc* (c-Myc T58A mutant) in *Nqo1-KD* Huh-7 cells, which revealed partial but significant rescue of cell proliferation (Fig 8A), colony formation (Fig 8B–C), and glycolytic gene expression (Fig 8D–E) in *Nqo1-KD* cells. These data indicate that Akt and c-Myc are

two of the main downstream targets through which Nqo1 controls metabolic reprogramming in liver cancer cells.

Discussion

Nqo1 is a cytosolic flavoprotein that utilizes NADH or NADPH to catalyze the reduction of quinones, thereby preventing the generation of free radicals. Hence, Nqo1 may protect cells from oxidative stress (34, 35). Accordingly, *Nqo1* deficiency in mice resulted in increased susceptibility to chemical-induced skin carcinogenesis and radiation-induced myeloproliferative disease (36, 37). However, a large number of studies demonstrated strong upregulation of Nqo1 in various cancers such as breast, pancreas, liver, bladder, ovary, thyroid, colorectal, cholangiocarcinoma, uterine cervical cancer, melanoma, and lung cancer (15–19). Moreover, *Nqo1-KD* inhibited proliferation of cholangiocarcinoma and adenocarcinoma cells (38, 39). Conversely, *Nqo1-OE* increased the proliferation of melanoma cells (40), suggesting that Nqo1 may function as an oncogene and promote cell proliferation and/or survival of certain cancer cells. Additionally, increased expression of Nqo1 protein was correlated with tumor size, advanced clinical stage, and decreased patient survival rates in breast, liver and lung cancers (17, 19, 20), signifying that Nqo1 may also be involved in the progression of certain cancers. In the current study, we demonstrate that Nqo1 is strongly overexpressed in mouse and human liver tumors and in liver cancer cell lines. *Nqo1-KD* significantly inhibited proliferation of liver cancer cells and xenograft tumor growth, and absence of *Nqo1* strongly inhibited DEN-induced HCC in mice. Our observations suggest that Nqo1 functions as an oncogene in HCC.

Most significantly, we discovered that Nqo1 is required for the activation of two major pro-oncogenic signaling pathways in liver cancer cells: PI3K/Akt and MAPK/ERK. These signaling pathways are often dysregulated and hyper-activated in HCC and promote cancer cell metabolic adaptation, proliferation and tumor growth (1, 6, 7, 33, 41–43). *Nqo1-KD* in liver cancer cells blunted activation of both the PI3K/Akt and MAPK/ERK signaling pathways, indicating that Nqo1 functions upstream and is required for the activation of these pathways. How does Nqo1 regulate these two pathways simultaneously? There is a substantial amount of cross-talk and critical regulatory points between the PI3K/Akt and MAPK/ERK pathways (22, 23, 25–28). Therefore, it is entirely possible that Nqo1 may target factors that connect these two pathways. Some of the factors that operate in the cross-regulation of the PI3K/Akt and MAPK/ERK pathways are PTEN, p53, AMPK, GSK3 β and PP2A (22, 23, 25–28). Very fascinatingly, we found that Nqo1 interferes with PP2A enzymatic activity which is one of the strongest molecular links between the PI3K/Akt and MAPK/ERK pathways (25–28). PP2A operates at the inter-junction of these two pathways and dephosphorylates and inactivates PDK1, Akt, Raf, MEK and ERK1/2, thereby shutting down the PI3K/Akt and MAPK/ERK pathways simultaneously. Therefore, factors that target PP2A apparently cause constitutive activation of both the PI3K/Akt and MAPK/ERK pathways, and many viral proteins target PP2A to constitutively activate both these pathways and promote tumorigenesis (44). *Nqo1-KD* increased PP2A activity, suggesting that PP2A is one of the major downstream targets of Nqo1. Therefore, it appears that Nqo1 exerts its influence on metabolic adaptation, cell proliferation and tumor growth via PP2A-linked signaling pathways such as the PI3K/Akt and MAPK/ERK pathways.

Our other substantial observation is that *Nqo1-KD* enhanced PTEN expression by promoting its transcription. One of the signaling pathways that regulate *Pten* transcription runs through ERK/CREB/c-Jun. ERK1/2 is known to phosphorylate and activate CREB, which can induce *c-Jun* expression. c-Jun directly binds to the *Pten* promoter and inhibits its transcription (30–32). Here, we provide evidence that *Nqo1-KD* enhanced PP2A activity, inhibited ERK and CREB activation, and suppressed c-Jun expression and its subsequent inhibitory binding to the *Pten* promoter, leading to enhanced expression of *Pten*. Inhibition of PP2A activity or re-expression of *Creb* in *Nqo1-KD* cells induced c-Jun expression and suppressed PTEN. Based on these observations, we propose that Nqo1 regulates *Pten* expression via the PP2A/ERK/CREB/c-Jun pathway. Overall, we propose Nqo1 as a novel player in the regulation of both the PI3K/Akt and MAPK pathways, and functions upstream to activate both of these pathways simultaneously (Fig 8F).

The down-stream effectors of the PI3K/Akt and MAPK/ERK pathways, Akt and c-Myc, play central roles in metabolic adaptation. Together Akt and c-Myc regulate the majority of the glycolytic and glutaminolysis genes whose expression and/or activation is essential for reprogramming of glucose and glutamine metabolism and uncontrolled proliferation of cancer cells (9–11, 33, 43). Our work here establishes Nqo1 as a key mediator of metabolic adaptation due to its tremendous influence on the activation of the PI3K/Akt and MAPK/ERK activity in cancer cells. Nqo1 appears to control glycolysis and glutaminolysis via two of the most powerful regulators of metabolic adaptation, PP2A and PTEN. For example, *Pten* elevation alone is sufficient to induce a tumor-suppressive, anti-Warburg state (13). We demonstrate that Nqo1 ablation not only induced PTEN expression and impaired activation of Akt, but also blunted activation of the MAPK/ERK pathway and downregulated c-Myc, leading to suppression of both glycolytic and glutaminolysis genes and reversal of the Warburg effect. Conversely, *Nqo1-OE* downregulated PTEN, activated Akt and induced expression of c-Myc, leading to enhanced expression of glycolytic and glutaminolysis genes and promotion of metabolic adaptation. Likewise, a recent study by metabolomics profiling showed that *Nqo1* depletion downregulates HK2 and suppresses glycometabolism in NSCLC cells, highlighting the involvement of Nqo1 in glucose metabolism (45). In conclusion, our findings that Nqo1 functions as an upstream activator of the PI3K/Akt and MAPK/ERK signaling pathways and regulates metabolic adaptation, HCC cell proliferation and tumor growth suggest that Nqo1 could function as a potential therapeutic target to inhibit HCC growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors are very grateful to Dr. Minho Shong, Korea Research Institute of Bioscience and Biotechnology, South Korea for providing *Nqo1*^{+/-} mouse frozen sperm. We also thank Dr. Angel Barco, The Instituto de Neurociencias de Alicante, for providing VP16-CREB plasmid and Dr. Rhea-Beth Markowitz for critically reviewing and editing the manuscript. We thank Jingling Yuan for animal care, and the Georgia Cancer Center Metabolic Resource for providing assistance with the Seahorse XF96 analyzer.

Financial Support

This research is supported by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) DP2DK105565 (Ade) of the National Institutes of Health.

Abbreviations

Nqo1	NAD(P)H Quinone Dehydrogenase 1
Nqo1-KD	Nqo1 knockdown
Nqo1-OE	Nqo1 overexpression
PI3K	Phosphoinositide 3-kinase
Akt	Akt serine/threonine kinase
MAPK	Mitogen activated protein kinase
ERK	Extracellular signal-regulated kinase
HCC	Hepatocellular carcinoma
PTEN	Phosphatase and tensin homolog
CREB	CAMP responsive element binding protein
c-Jun	Jun proto-oncogene
OXPHOS	Oxidative phosphorylation
HK1	Hexokinase 1
PFK1	Phosphofructokinase 1
LDH	Lactate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase 1
SLC38A5	Solute carrier family 38 member 5
SLC1A5	Solute carrier family 1 member 5
GLS	Glutaminase
TCA cycle	Tricarboxylic acid cycle
α-KG	alpha-ketoglutarate
HK2	Hexokinase 2
PFK	Phosphofructokinase
ENO1	Enolase 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
PGK1	Phosphoglycerate kinase 1

LDHA	Lactate dehydrogenase A
Glut 1	Glucose transporter 1
PP2A	Protein phosphatase 2A
DEN	diethylnitrosamine
NADPH	Nicotinamide adenine dinucleotide phosphate
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
8-OHdG	8-hydroxy-2-deoxyguanosine
mTOR	The mechanistic target of rapamycin
GSK3β	Glycogen synthase kinase-3 beta
EGFR	Epidermal growth factor receptor
PPARγ	Peroxisome proliferator activated receptor gamma
TGFβ	Transforming growth factor beta
NFκB	Nuclear factor kappa B subunit 1
Hes1	Hes family BHLH transcription factor 1
OCR	Oxygen consumption rate
ECAR	Extracellular acidification rate
Cyt C	Cytochrome C
FDR	False discovery rate
IHC	Immunohistochemistry
H&E	Hematoxylin and eosin
ChIP	Chromatin immunoprecipitation
PCNA	Proliferating cell nuclear antigen
CoxIV	cytochrome c oxidase subunit IV

References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–674. [PubMed: 21376230]
2. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab* 2008;7:11–20. [PubMed: 18177721]
3. Dang CV. Links between metabolism and cancer. *Genes Dev* 2012;26:877–890. [PubMed: 22549953]

4. Levine AJ, Puzio-Kuter AM. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. *Science* 2010;330:1340–1344. [PubMed: 21127244]
5. Hensley CT, Wasti AT, DeBerardinis RJ. Glutamine and cancer: cell biology, physiology, and clinical opportunities. *J Clin Invest* 2013;123:3678–3684. [PubMed: 23999442]
6. Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. *Cancer Cell* 2012;21:297–308. [PubMed: 22439925]
7. Pavlova NN, Thompson CB. The Emerging Hallmarks of Cancer Metabolism. *Cell Metab* 2016;23:27–47. [PubMed: 26771115]
8. Gottlob K, Majewski N, Kennedy S, Kandel E, Robey RB, Hay N. Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev* 2001;15:1406–1418. [PubMed: 11390360]
9. Miller DM, Thomas SD, Islam A, Muench D, Sedoris K. c-Myc and cancer metabolism. *Clin Cancer Res* 2012;18:5546–5553. [PubMed: 23071356]
10. Pelengaris S, Khan M, Evan G. c-MYC: more than just a matter of life and death. *Nat Rev Cancer* 2002;2:764–776. [PubMed: 12360279]
11. Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, Zeller KI, et al. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* 2009;458:762–765. [PubMed: 19219026]
12. Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang XY, Pfeiffer HK, Nissim I, et al. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc Natl Acad Sci U S A* 2008;105:18782–18787. [PubMed: 19033189]
13. Garcia-Cao I, Song MS, Hobbs RM, Laurent G, Giorgi C, de Boer VC, Anastasiou D, et al. Systemic elevation of PTEN induces a tumor-suppressive metabolic state. *Cell* 2012;149:49–62. [PubMed: 22401813]
14. Floter J, Kaymak I, Schulze A. Regulation of Metabolic Activity by p53. *Metabolites* 2017;7.
15. Siegel D, Franklin WA, Ross D. Immunohistochemical detection of NAD(P)H:quinone oxidoreductase in human lung and lung tumors. *Clin Cancer Res* 1998;4:2065–2070. [PubMed: 9748120]
16. Schlager JJ, Powis G. Cytosolic NAD(P)H:(quinone-acceptor)oxidoreductase in human normal and tumor tissue: effects of cigarette smoking and alcohol. *Int J Cancer* 1990;45:403–409. [PubMed: 2307529]
17. Yang Y, Zhang Y, Wu Q, Cui X, Lin Z, Liu S, Chen L. Clinical implications of high NQO1 expression in breast cancers. *J Exp Clin Cancer Res* 2014;33:14. [PubMed: 24499631]
18. Ma Y, Kong J, Yan G, Ren X, Jin D, Jin T, Lin L, et al. NQO1 overexpression is associated with poor prognosis in squamous cell carcinoma of the uterine cervix. *BMC Cancer* 2014;14:414. [PubMed: 24912939]
19. Li Z, Zhang Y, Jin T, Men J, Lin Z, Qi P, Piao Y, et al. NQO1 protein expression predicts poor prognosis of non-small cell lung cancers. *BMC Cancer* 2015;15:207. [PubMed: 25880877]
20. Lin L, Sun J, Tan Y, Li Z, Kong F, Shen Y, Liu C, et al. Prognostic implication of NQO1 overexpression in hepatocellular carcinoma. *Hum Pathol* 2017;69:31–37. [PubMed: 28964792]
21. Sears RC. The life cycle of C-myc: from synthesis to degradation. *Cell Cycle* 2004;3:1133–1137. [PubMed: 15467447]
22. Mendoza MC, Er EE, Blenis J. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends Biochem Sci* 2011;36:320–328. [PubMed: 21531565]
23. Aksamitiene E, Kiyatkin A, Kholodenko BN. Cross-talk between mitogenic Ras/MAPK and survival PI3K/Akt pathways: a fine balance. *Biochem Soc Trans* 2012;40:139–146. [PubMed: 22260680]
24. Nakdimon I, Walser M, Frohli E, Hajnal A. PTEN negatively regulates MAPK signaling during *Caenorhabditis elegans* vulval development. *PLoS Genet* 2012;8:e1002881. [PubMed: 22916028]
25. Letourneux C, Rocher G, Porteu F. B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. *EMBO J* 2006;25:727–738. [PubMed: 16456541]

26. Ugi S, Imamura T, Ricketts W, Olefsky JM. Protein phosphatase 2A forms a molecular complex with Shc and regulates Shc tyrosine phosphorylation and downstream mitogenic signaling. *Mol Cell Biol* 2002;22:2375–2387. [PubMed: 11884620]
27. Kumar A, Pandurangan AK, Lu F, Fyrst H, Zhang M, Byun HS, Bittman R, et al. Chemopreventive sphingadienes downregulate Wnt signaling via a PP2A/Akt/GSK3beta pathway in colon cancer. *Carcinogenesis* 2012;33:1726–1735. [PubMed: 22581840]
28. Kiely M, Kiely PA. PP2A: The Wolf in Sheep's Clothing? *Cancers (Basel)* 2015;7:648–669. [PubMed: 25867001]
29. Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* 2001;353:417–439. [PubMed: 11171037]
30. Hettinger K, Vikhanskaya F, Poh MK, Lee MK, de Belle I, Zhang JT, Reddy SA, et al. c-Jun promotes cellular survival by suppression of PTEN. *Cell Death Differ* 2007;14:218–229. [PubMed: 16676006]
31. Bermudez Brito M, Goulielmaki E, Papakonstanti EA. Focus on PTEN Regulation. *Front Oncol* 2015;5:166. [PubMed: 26284192]
32. Lopez-Bergami P, Huang C, Goydos JS, Yip D, Bar-Eli M, Herlyn M, Smalley KS, et al. Rewired ERK-JNK signaling pathways in melanoma. *Cancer Cell* 2007;11:447–460. [PubMed: 17482134]
33. Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, Zhuang H, et al. Akt stimulates aerobic glycolysis in cancer cells. *Cancer Res* 2004;64:3892–3899. [PubMed: 15172999]
34. Ross D, Kepa JK, Winski SL, Beall HD, Anwar A, Siegel D. NAD(P)H:quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms. *Chem Biol Interact* 2000;129:77–97. [PubMed: 11154736]
35. Nebert DW, Roe AL, Vandale SE, Bingham E, Oakley GG. NAD(P)H:quinone oxidoreductase (NQO1) polymorphism, exposure to benzene, and predisposition to disease: a HuGE review. *Genet Med* 2002;4:62–70. [PubMed: 11882782]
36. Iskander K, Gaikwad A, Paquet M, Long DJ 2nd, Brayton C, Barrios R, Jaiswal AK. Lower induction of p53 and decreased apoptosis in NQO1-null mice lead to increased sensitivity to chemical-induced skin carcinogenesis. *Cancer Res* 2005;65:2054–2058. [PubMed: 15781611]
37. Iskander K, Barrios RJ, Jaiswal AK. Disruption of NAD(P)H:quinone oxidoreductase 1 gene in mice leads to radiation-induced myeloproliferative disease. *Cancer Res* 2008;68:7915–7922. [PubMed: 18829548]
38. Madajewski B, Boatman MA, Chakrabarti G, Boothman DA, Bey EA. Depleting Tumor-NQO1 Potentiates Anoikis and Inhibits Growth of NSCLC. *Mol Cancer Res* 2016;14:14–25. [PubMed: 26553038]
39. Butsri S, Kukongviriyapan V, Senggunprai L, Kongpetch S, Zeekpudsa P, Prawan A. Downregulation of NAD(P)H:quinone oxidoreductase 1 inhibits proliferation, cell cycle and migration of cholangiocarcinoma cells. *Oncol Lett* 2017;13:4540–4548. [PubMed: 28599455]
40. Garate M, Wani AA, Li G. The NAD(P)H:Quinone Oxidoreductase 1 induces cell cycle progression and proliferation of melanoma cells. *Free Radic Biol Med* 2010;48:1601–1609. [PubMed: 20226854]
41. Zhou Q, Lui VW, Yeo W. Targeting the PI3K/Akt/mTOR pathway in hepatocellular carcinoma. *Future Oncol* 2011;7:1149–1167. [PubMed: 21992728]
42. Yang S, Liu G. Targeting the Ras/Raf/MEK/ERK pathway in hepatocellular carcinoma. *Oncol Lett* 2017;13:1041–1047. [PubMed: 28454211]
43. Jones RG, Thompson CB. Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev* 2009;23:537–548. [PubMed: 19270154]
44. Guernnon J, Godet AN, Galioot A, Falanga PB, Colle JH, Cayla X, Garcia A. PP2A targeting by viral proteins: a widespread biological strategy from DNA/RNA tumor viruses to HIV-1. *Biochim Biophys Acta* 2011;1812:1498–1507. [PubMed: 21856415]
45. Cheng X, Liu F, Liu H, Wang G, Hao H. Enhanced glycometabolism as a mechanism of NQO1 potentiated growth of NSCLC revealed by metabolomic profiling. *Biochem Biophys Res Commun* 2018;496:31–36. [PubMed: 29291405]

Figure 1. Upregulation of Nqo1 in HCC tissues and in cell lines.

(A) Heat map. RNA sequencing analysis of mouse non-tumorous (NT) versus liver tumor (T) tissues (n=3), Green – downregulated and Red – upregulated genes. A 4-fold differential expression with a false discovery rate (FDR) of 0.001 were applied. With that criteria, 318 genes were downregulated and 648 genes were upregulated in liver tumors compared to normal livers. (B) Magnified heat map showing *Nqo1* differential expression in 3 independent liver tumors compared to normal livers. (C) *Nqo1* expression is 14.64 fold upregulated in liver tumors compared to non-tumorous tissues. (D) Western blot showing the expression level of Nqo1 in normal mouse liver and in DEN-induced liver tumors, (n=5). (E) Expression of Nqo1 protein in primary hepatocyte cell line H2.35 and human liver cancer cell lines HepG2 and Huh-7. (F–G) Representative Nqo1 IHC showing no staining on human non-tumorous tissues (F) but strong intense cytoplasmic staining (G) in HCC samples. Approximately 60% of HCC samples stained strongly positive for Nqo1. (H)

Representative human HCC sample showing no Nqo1 staining. Approximately 40% of HCC samples displayed very low to no detectable Nqo1 staining. Scale bar 300µm. **(I–J)** Representative Nqo1 IHC staining in paired human HCC and surrounding normal liver tissue. Liver tumor tissues (T) showed strong Nqo1 staining whereas non-tumorous (NT) areas are mostly negative. Scale bar 200µm. **(K)** Nqo1 staining intensity was determined semi-quantitatively and was graded as absent (0), weak (1), strong (2) and very strong (3), n=14, *** $p<0.0005$. **(L–M)** Representative Nqo1 IHC showing weak staining in the sinusoidal cells of mouse non-tumorous tissues (L) but strong intense cytoplasmic staining (M) in all DEN-induced HCC samples, n=6. Scale bar 100µm.

Figure 2. Nqo1 is required for liver cancer cell proliferation, colony formation and xenograft growth

(A) Western blot showing the expression of Nqo1 protein in *scramble* and *Nqo1* knockdown (*Nqo1-KD*) Huh-7 cells. Out of 5 *shRNAs* tested, 3 *shRNAs* efficiently knocked down *Nqo1*. *shRNA-1* was used to generate *Nqo1-KD* stable cells. (B) Relative proliferation of *Nqo1-KD* Huh-7 cells was significantly reduced compared to control cells as measured by cell counting method for 6 days, $n=3$, * $p<0.05$, ** $p<0.005$, *** $p<0.0005$. (C–D) Representative photos showing the migration (C) and migration area (D) of *scramble* and *Nqo1-KD* Huh-7 cells, $n=3$, ** $p<0.005$, *** $p<0.0005$. (E–F) Crystal violet-stained colonies formed by control and *Nqo1-KD* Huh-7 cells 21 days after plating (E); colony formation ability of *Nqo1-KD* cells was significantly impaired compared to control cells (F), $n=3$, ** $p<0.005$. (G) Representative photos showing significantly reduced xenograft tumor growth of *Nqo1-KD* Huh-7 cells compared to control cells. (H) Western blot showing the expression level of Nqo1 in *scramble* and *Nqo1-KD* xenografts. (I–J) Xenograft tumor

volume (I) and tumor weight (J) were significantly reduced in *Nqo1-KD* cells compared to *scramble* cells, n=5, *** $p<0.0005$. All the quantitative data are represented as mean $\pm$ SD.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. DEN-induced HCC is inhibited in *Nqo1*^{-/-} mice

(A) Representative photographs showing liver tumor growth in *Nqo1*^{+/+} and *Nqo1*^{-/-} mice 8 months after DEN treatment. (B) Body weight, (C) average number of tumors per liver, (D) Percentage of tumor incidence and liver tumor size and (E) Liver/body weight ratio in *Nqo1*^{+/+} and *Nqo1*^{-/-} mice. Liver tumor size in (D) was categorized into 3 groups; small (<2mm), medium (2–5mm) and large (>5mm), n=9, **p<0.005, ***p<0.0005. (F–I) Representative H&E (F), Oil-Red-O (G), and Sirius-Red (H) stained images of *Nqo1*^{+/+} and *Nqo1*^{-/-} mouse livers 8 months after DEN treatment. (I) IHC staining of PCNA on the liver tumors of *Nqo1*^{+/+} and *Nqo1*^{-/-} mice. Insert showing the percentage of PCNA-positive cells in the liver tumors of *Nqo1*^{+/+} and *Nqo1*^{-/-} mice, (n=9, ***p<0.0005). All the quantitative data are represented as mean ± SD.

Figure 4. Enhanced PP2A activity and inactivation of PI3K/Akt and MAPK/ERK pathways in response to *Nqo1*-KD

(A) Western blots showing expression and activity levels of the indicated proteins in *scramble* and *Nqo1*-KD Huh-7 cells. (B) PIP2 levels in *scramble* and *Nqo1*-KD Huh-7 cells, $n=3$, $**p<0.005$. (C) Western blots showing expression and activity status of the indicated proteins in *scramble* and *Nqo1*-KD Huh-7 cells. (D) Western blots showing the expression and activity levels of the indicated proteins in *Nqo1*^{+/+} and *Nqo1*^{-/-} mouse liver tumors. (E) Expression levels of p53, AMPK and the sub-units of PP2A in *scramble* and *Nqo1*-KD cells. (F) PP2A enzymatic activity in *scramble* and *Nqo1*-KD Huh-7 cells, $n=4$, $***p<0.0005$. (G) PP2A enzymatic activity in *scramble* and *Nqo1*-KD Huh-7 cells after treatment with 10μM of PP2A inhibitor LB-100 for 48 h, $n=3$, $*p<0.05$, $**p<0.005$. (H) Western blots showing the expression and activity levels of the indicated proteins in *scramble* and *Nqo1*-KD Huh-7 cells after treatment with 10μM of LB-100 for 48 h. Red * in Western blots indicates strong detectable difference. All the quantitative data are represented as mean $\pm$ SD.

Figure 5. Nqo1 regulates *Pten* expression via ERK/CREB/c-Jun pathway

(A) Relative expression level of *Pten* in control and *Nqo1-KD* Huh-7 cells, $n=3$, $**p<0.005$. (B) Western blots showing expression levels of the indicated proteins in *scramble* and *Nqo1-KD* Huh-7 cells. (C) According to our proposed model, Nqo1 inhibits or interferes with PP2A activity, leading to constitutive activation of ERK1/2, which phosphorylates and activates CREB. Activated CREB induces c-Jun transcription. c-Jun directly binds to the *Pten* promoter and suppresses its transcription. (D) Expression levels of indicated proteins in *scramble* and *Nqo1-KD* Huh-7 cells. (E) Expression levels of the indicated proteins in *Nqo1-KD* Huh-7 cells after treatment with $10\mu\text{M}$ LB-100 for 48h. (F) ChIP-qPCR analysis of c-Jun binding to the *Pten* promoter in *scramble* and *Nqo1-KD* Huh-7 cells after normalizing to 18S binding, $n=3$, $**p<0.005$. (G) Western blots showing expression levels of the indicated proteins after expressing vector control or constitutively active form of *Creb* (VP16-CREB) in *Nqo1-KD* Huh-7 cells. (H) Relative mRNA expression level of *Pten* in

Nqo1^{+/+} and *Nqo1*^{-/-} mouse liver tumors. n=6, **p<0.005. **(I)** Expression levels of the indicated proteins in *Nqo1*^{+/+} and *Nqo1*^{-/-} liver tumors. **(J)** IHC showing strong intense PTEN staining in *Nqo1*^{-/-} mouse liver tumor tissues, whereas PTEN expression is very low to undetectable in *Nqo1*^{+/+} liver tumors. **(K)** Relative mRNA expression level of *Pten* in *Nqo1*^{+/+} and *Nqo1*^{-/-} normal mouse livers, n=4, **p<0.005. **(L)** Expression level of PTEN in *Nqo1*^{+/+} and *Nqo1*^{-/-} normal mouse livers. Red * in Western blots indicates strong detectable difference. All the quantitative data are represented as mean ± SD.

Figure 6. *Nqo1*-KD caused downregulation of glycolysis and glutaminolysis genes and suppressed aerobic glycolysis

(A) Glucose uptake in control and *Nqo1*-KD Huh-7 cells, $n=3$, *** $p<0.0005$. (B) Cellular lactate levels in control and *Nqo1*-KD Huh-7 cells, $n=3$, *** $p<0.0005$. (C–D) Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of control and *Nqo1*-KD cells measured by Seahorse Bioscience XF96 analyzer, $n=3$, ** $p<0.005$, *** $p<0.0005$. (E) Representative flow cytometry analysis showing no significant difference in mitochondrial content between control and *Nqo1*-KD cells. $n=3$. (F–G) Relative mRNA expression levels of glycolytic genes (F), and glutamine transporters (G) in control and *Nqo1*-KD cells, $n=3$, * $p<0.05$, ** $p<0.005$. (H) Western blots showing expression and activity levels of the indicated proteins in *scramble* and *Nqo1*-KD Huh-7 cells. (I) Hexokinase activity levels in control and *Nqo1*-KD cells, $n=3$, ** $p<0.005$. (J) Expression levels of the indicated proteins in *Nqo1*^{+/+} and *Nqo1*^{-/-} liver tumors. (K–L) Glucose and lactate levels in *Nqo1*^{+/+} and *Nqo1*^{-/-} liver tumors, $n=6$, ** $p<0.005$. Red * in Western blots

indicates strong detectable difference. All the quantitative data are represented as mean $\pm$ SD.

Figure 7. *Nqo1-OE* caused upregulation of glycolysis and glutaminolysis genes and enhanced aerobic glycolysis

(A) Proliferation rate of *Nqo1-OE* Huh-7 cells was significantly increased compared to control cells as measured by cell counting method for 6 days, $n=3$, $*p<0.05$, $**p<0.005$. (B) Glucose uptake in control and *Nqo1-OE* Huh-7 cells, $n=3$, $**p<0.005$. (C) Cellular lactate levels in control and *Nqo1-OE* cells, $n=3$, $**p<0.005$. (D-E) Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of control and *Nqo1-OE* Huh-7 cells measured by Seahorse Bioscience XF96 analyzer. $n=3$, $**p<0.005$. (F) Western blots showing expression and activity levels of indicated proteins in control and *Nqo1-OE* Huh-7 cells. (G-H) Western blots showing expression and activity levels of the indicated proteins in control and *Nqo1-OE* H2.35 cells. (I) Glucose uptake in control and *Nqo1-OE* H2.35 cells, $n=3$, $*p<0.05$. (J) Cellular lactate levels in control and *Nqo1-OE* cells, $n=3$, $*p<0.05$. (K-L) Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of control and *Nqo1-OE* H2.35 cells measured by Seahorse Bioscience XF96 analyzer, $n=3$, $*p<0.05$.

** $p < 0.005$. Red * in Western blots indicates strong detectable difference. All the quantitative data are represented as mean $\pm$ SD.

Figure 8. Forced expression of Akt and c-Myc partially reverse *Nqo1-KD*-associated phenotypes Proliferation rate of indicated cell lines as measured by cell counting method for 6 days, $n=3$, $*p<0.05$, $**p<0.005$. (B–C) Crystal violet-stained colonies formed by indicated cell lines 21 days after plating (B); colony formation ability of *Nqo1-KD* cells was partially but significantly rescued when constitutively active forms of *Akt* or *c-Myc* were re-expressed (C), $n=3$, $**p<0.005$, $***p<0.0005$. (D–E) Western blots showing the expression levels of glycolytic proteins after expressing vector control or constitutively active forms of *Akt* or *c-Myc* in *Nqo1-KD* Huh-7 cells. Red * in Western blots indicates strong detectable difference. All the quantitative data are represented as mean $\pm$ SD. (F) Proposed model: PP2A functions as one of the central players in the regulation of both the PI3K/Akt and MAPK/ERK pathways. PP2A can dephosphorylate PDK1, Akt, Raf and ERK1/2, thereby shutting down both the PI3K/Akt and MAPK/ERK pathways. PP2A also dephosphorylates and activates GSK3 β leading to GSK3 β -mediated phosphorylation and degradation of c-Myc. In the

presence of Nqo1, Nqo1 interferes with PP2A, thereby preventing it from dephosphorylating and inactivating protein kinases in the PI3K/Akt and MAPK/ERK pathways, leading to their constitutive activation. Activated ERK1/2 also suppresses *Pten* expression via the CREB/c-Jun pathway, resulting in further activation of the PI3K/Akt pathway. Activated Akt and ERK1/2 phosphorylate and inactivate GSK3 β , thereby preventing GSK3 β -mediated phosphorylation and degradation of c-Myc. ERK1/2 can also directly phosphorylate and stabilize c-Myc. Akt and c-Myc together regulate the expression of vast majority of glycolysis and glutaminolysis genes, promoting metabolic adaptation, proliferation and tumor growth. **In the absence of Nqo1**, activated PP2A dephosphorylates kinases in both the PI3K/Akt and MAPK/ERK pathways, leading to simultaneously shutting down of both pathways. Due to ERK1/2 inactivation and impaired activation of the CREB/c-Jun pathway, *Pten* is upregulated. PTEN, by targeting PIP3 further inhibits PI3K/Akt signaling. Overall, due to impaired activation of Akt and downregulation of c-Myc, glycolysis and glutaminolysis genes are not induced, leading to blockade of metabolic adaptation, proliferation and tumor growth. Green and Red arrows represent up- and down-regulation of indicated proteins in response to the presence or absence of Nqo1.