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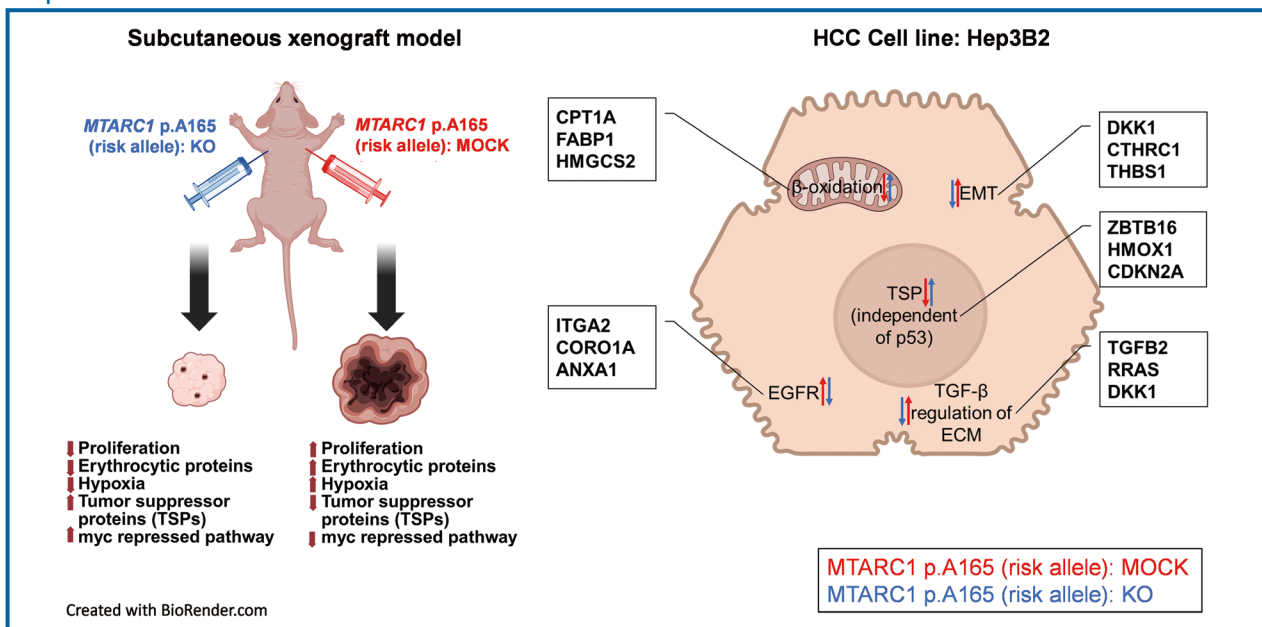
Original Article

MTARC1 p.A165 ablation reduces hepatocellular carcinoma aggressiveness in vitro and in vivo

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Graphical Abstract



Study Highlights

- Downregulation of the *MTARC1* p.A165 risk allele reduces cell proliferation, intracellular neutral lipid accumulation, and migration, while enhancing β -oxidation in Hep3B2 cells.
- Transient knockdown of *CPT1A*, a key protein involved in β -oxidation, partially reverses the beneficial effects observed upon *MTARC1* p.A165 knockout.
- *MTARC1* p.A165 knockout markedly reduces tumor growth and proliferation in a subcutaneous HCC xenograft model.
- These findings identify *MTARC1* as a potential therapeutic target in hepatocellular carcinoma.

Background/Aims: Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related mortality worldwide and is driven by metabolic reprogramming that supports tumor growth and progression. A common missense genetic variant (rs2642438, p.A165T) in mitochondrial amidoxime reducing component 1 (*MTARC1*), identified as protective against liver disease, has been recently associated with lower prevalence of steatosis, cirrhosis, and HCC. However, the mechanistic role of *MTARC1* in HCC is unclear. Therefore, we sought to decipher the role of *MTARC1* in HCC.

Methods: We investigated the role of *MTARC1* in HCC by performing siRNA-mediated knockdown across human immortalized HCC cell lines (Hep3B2, HuH7, HepG2 and HepaRG) homozygous for the risk allele (p.A165) and by generating stable CRISPR-Cas9 knockout (KO) models. Next, we assessed the effect of *MTARC1* loss on cell proliferation, migration, lipid metabolism, and fatty acid oxidation in vitro, as well as tumor aggressiveness in a subcutaneous xenograft mouse model. Additionally, we performed global proteomics in both in vitro and xenograft models.

Results: Transient knockdown of *MTARC1* p.A165 reduced proliferation in HCC cell lines. CRISPR-Cas9-mediated stable *MTARC1* p.A165 KO in Hep3B2 cells led to decreased neutral lipid intracellular accumulation, enhanced β -oxidation and reduced cell migration. An *MTARC1* KO xenograft model had reduced tumor volume. Proteomic analyses of both in vitro HCC cells and xenograft tumors revealed inhibition of oncogenic pathways and activation of anti-proliferative proteins.

Conclusions: Downregulation of *MTARC1* p.A165 inhibits lipid accumulation, dampens tumor-promoting pathways and restricts tumor growth, highlighting *MTARC1* as a promising therapeutic target for HCC. (*Clin Mol Hepatol* 2026;32:829-842)

Keywords: *MTARC1*; Subcutaneous xenograft model; Hypoxia; EMT; Fatty acid degradation

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of liver cancer and the third leading cause of cancer-related mortality worldwide.¹ The incidence of HCC continues to rise, mainly due to increasing prevalence of chronic

liver diseases, such as metabolic dysfunction-associated steatotic liver disease (MASLD) and alcohol-related liver disease.² Despite recent advances in its treatment regimen, including immune checkpoints and tyrosine kinase inhibitors, the overall prognosis for HCC remains poor, with a median survival of less than two years, indicating an un-

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Abbreviations:

ALDOA, aldolase A; BSA, bovine serum albumin; CDH6, cadherin 6; CDKN1B, cyclin-dependent kinase inhibitor 1B; CDKN2A, cyclin-dependent kinase inhibitor 2A; CID, collision-induced dissociation (MS fragmentation); CNX, calnexin; CPT1a, carnitine palmitoyltransferase 1A; CTHRC1, collagen triple helix repeat containing 1; DAPI, 4',6-diamidino-2-phenylindole; DKK1, Dickkopf-related protein 1; EGFR, epidermal growth factor receptor; EFNA1, ephrin-A1; EMT, epithelial-mesenchymal transition; FAIMS, field asymmetric ion mobility spectrometry; FDR, false discovery rate; FOXO3, forkhead box O3; GALK1, galactokinase 1; GWAS, genome-wide association studies; HBA1, HBB, hemoglobin subunit alpha 1, hemoglobin subunit beta; HCC, hepatocellular carcinoma; HCD, higher-energy collision dissociation (MS fragmentation); HEK293T, human embryonic kidney 293T cells; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hep3B2, hepatocellular carcinoma, 3B2; HepG2, hepatocellular carcinoma, G2; HepaRG, hepatocyte-related progenitor-derived RG cells; HEXIM1, hexamethylene bis-acetamide inducible 1; HuH7, human hepatoma 7; KO, knockout; LC-MS3, liquid chromatography coupled to triple-stage mass spectrometry; MARC1 / *MTARC1*, mitochondrial amidoxime reducing component 1; MASLD, metabolically dysfunction associated steatotic liver disease; MS/MS, tandem mass spectrometry; NDRG1, N-myc down regulated Gene1; ORO, Oil Red O; PA, palmitate; PBS, phosphate-buffered saline; PGAM2, phosphoglycerate mutase 2; PRKAB1, protein kinase AMP-activated non-catalytic subunit beta 1; SD, standard deviation; siRNA, small interfering RNA; SMAD2, mothers against decapentaplegic homolog 2; SP3, single-pot, solid-phase-enhanced sample preparation (proteomics workflow); TGF- β , transforming growth factor beta; THBS1, thrombospondin-1; TMT, tandem mass tag (isobaric labeling for proteomics); ZFP36L1, zinc finger protein 36, C3H type-like 1

met medical need.^{3,4}

Metabolic reprogramming is a classical hallmark of HCC. HCC cells adapt their metabolic state to increase proliferation, evade cell death and survive under hypoxic conditions.⁵ This adaptation involves lipid accumulation through increased synthesis and impaired export, while altered fatty acid oxidation supports energy production and survival under stress.^{5,6} In this context, β -oxidation represents a double-edged metabolic pathway. On the one hand, enhanced β -oxidation may promote tumorigenic activity by providing energy and supporting survival,⁶ but on the other, it may exert anti-tumorigenic effects by limiting cell proliferation, such as in HCC, prostate and breast cancer, underscoring a context-dependent role of lipid metabolism in tumor progression.⁶ Understanding the molecular mediators of these processes is essential for identifying novel therapeutic targets.

Mitochondrial amidoxime reducing component 1 (MTARC1, also referred to as MARC1) is a molybdenum-containing enzyme present on the outer mitochondrial membrane.⁷ Initially characterized for its role in the reductive metabolism of xenobiotics, MTARC1 has subsequently been implicated in lipid metabolism and redox homeostasis.^{7,8} Genome-wide association studies (GWASs) have identified a common protective *MTARC1* variant (p.A165T) in which the minor allele is associated with lower hepatic triglyceride content, as well as a lower prevalence of cirrhosis and of HCC⁸⁻¹⁰ suggesting a relevant role of MTARC1 in cancer biology. However, the mechanistic contribution of MTARC1 to HCC biology has not been elucidated.

To address this gap, we investigated the role of MTARC1 in HCC using in vitro and in vivo models. We found that loss of *MTARC1* p.A165 inhibits HCC cell proliferation and disrupts key oncogenic signaling pathways. Further, in a subcutaneous xenograft model of *MTARC1* p.A165 knock-out (KO) cells in mice we found reduced tumor volume and weight compared with xenografts derived from *MTARC1* control (mock) cells. Together, these findings establish that downregulation of hepatic *MTARC1* p.A165 may represent a promising strategy to treat HCC.

MATERIALS AND METHODS

Details for each experiment are provided in the Support-

ing Information.

RESULTS

Transient downregulation of the *MTARC1* p.A165 risk allele reduces cellular proliferation in HCC cell lines

To investigate the role of *MTARC1* in HCC, we transiently downregulated its expression using siRNA in four human HCC cell lines homozygous for the p.A165 risk allele, namely, Hep3B2, Huh7, HepG2, and HepaRG. *MTARC1* p.A165 knockdown efficiency was confirmed by qPCR in all cell lines, with mRNA levels lower by more than 80% at 24 hours, 48 hours and 72 hours compared with scrambled siRNA control (siSCR) (Fig. 1, left panels; $P < 0.001$ for all time points).

MTARC1 p.A165 downregulation lowered the cell count by 34% in Hep3B2, 24% in Huh7 and 24% in HepG2 cells, respectively, averaged across the three time points (Fig. 1A–C, right panels; $P \leq 0.01$). In HepaRG cells, proliferation was reduced by 16% at 48 hours ($P \leq 0.01$) and at 72 hours ($P < 0.001$) (Fig. 1D, right panel).

These findings demonstrate that transient downregulation of *MTARC1* p.A165 exerts an anti-proliferative effect.

Stable KO of *MTARC1* p.A165 reduces intracellular neutral lipid content, cell proliferation and migration in vitro

To further characterize the role of MTARC1 in HCC, we generated a stable *MTARC1* p.A165 KO cell line using the CRISPR-Cas9 method in Hep3B2 cells and tested the effect of the KO on intracellular neutral lipid content, cell proliferation and migration in vitro. By immunoblot analysis we found a marked reduction of MTARC1 protein expression in the KO cell lysates (Fig. 2A, upper panel), and densitometric quantification normalized to calnexin (CNX) revealed a more than 90% reduction in MTARC1 protein levels ($P \leq 0.01$).

We found that *MTARC1* p.A165 KO cells had lower intracellular neutral lipid content, as visualized by Oil-Red-O staining (Fig. 2B; $P < 0.001$) compared with mock cells. Additionally, via a [³H]-palmitate oxidation assay we found higher

β -oxidation activity in KO cells compared with mock cells ($P \leq 0.01$), indicating higher fatty acid oxidation (Fig. 2D).

When we analyzed cell proliferation, we found that *MTARC1* p.A165 KO had lower proliferation compared with mock cells at 24 hours (35% reduction, $P < 0.001$), 48 hours (41.0% reduction, $P \leq 0.01$) and 72 hours (43% reduction, $P < 0.001$) post-seeding (Fig. 2C).

Next, we examined cell migration using a wound healing assay. Compared with mock cells, *MTARC1* p.A165 KO showed a markedly lower wound closure at 24 hours (73% inhibition, $P < 0.001$), 48 hours (55% inhibition, $P \leq 0.01$) and 72 hours (60% inhibition, $P \leq 0.01$) (Fig. 2E, lower panel).

These data were virtually identical in two different *MTARC1* mock and KO cell clones (Supplementary Fig. 1).

In vitro proteomic profiling reveals that *MTARC1* p.A165 KO inhibits oncogenic pathways in HCC

To gain further insight into the molecular mechanisms of *MTARC1* in HCC, we performed whole-cell proteomics on mock and *MTARC1* p.A165 KO Hep3B2 cells. We detected a total of 9,193 proteins, of which 238 were upregulated and 190 were downregulated (FDR adjusted, $\log_2 \text{FC} \geq \pm 0.58$) in *MTARC1* p.A165 KO cells compared with mock cells. To decipher the biological significance of these differentially expressed proteins, we next performed pathway enrichment

analysis and found that *MTARC1* p.A165 KO results in a profound downregulation of epithelial-mesenchymal transition (EMT), hypoxia, epidermal growth factor receptor 1 (EGFR1) signaling and TGF- β regulation of extracellular matrix, as well as upregulation of fatty acid metabolism and p53 signaling (Fig. 3). After analysis of the differentially expressed proteins, five proteins were randomly selected whose expression levels were unchanged between Hep3B2 mock and Hep3B2 *MTARC1* p.A165 KO cells (Supplementary Fig. 2A).

EMT enables cancer cells to acquire migratory and invasive properties.¹¹ We found that levels of CTHRC1, THBS1, CDH6 and DKK1 were lower in *MTARC1* p.A165 KO cells compared with mock cells, resulting in impaired mesenchymal transition. This suggests that the loss of *MTARC1* p.A165 hinders the epithelial-to-mesenchymal plasticity of HCC cells (Fig. 3A).

Hypoxia is a major hallmark of HCC, driving tumor aggressiveness, metabolic reprogramming and extracellular matrix remodeling.¹² We found that the expression levels of proteins, such as BGN, NDRG1 and ALDOA, were markedly lower in the *MTARC1* p.A165 KO cells compared with mock cells (Fig. 3B).

The EGFR pathway is a critical driver of HCC progression¹³ and *MTARC1* p.A165 KO led to lower levels of various EGFR-associated regulators, including ITGA2, TAGLN and TGFB2, compared with mock cells (Fig. 3C).

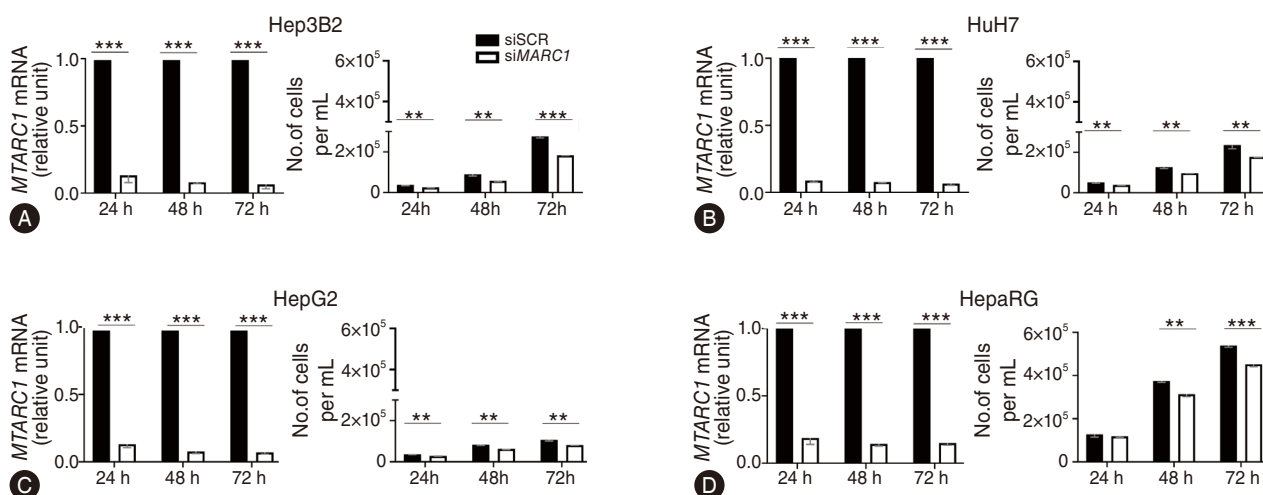


Figure 1. *MTARC1* p.A165 risk allele downregulation reduces cell proliferation. (A–D) mRNA expression levels of *MTARC1* (left panels) and cell count per mL (right panels) assessed in (A) Hep3B2, (B) HuH7, (C) HepG2 and (D) HepaRG cells after siRNA mediated knock-down of *MTARC1*. Cells were transfected with either siSCR (control) or si*MTARC1*, and both mRNA expression and cell count was measured at 24 hours, 48 hours, and 72 hours post-transfection. *P*-values were calculated using unpaired Student's *t*-test. Data are shown as mean $\pm$ SD for three independent experiments. Statistical analysis was performed using GraphPad Prism version 9 (San Diego, CA, USA). ** $P < 0.01$, *** $P < 0.001$. *MTARC1*, mitochondrial amidoxime-reducing component 1; siSCR, scrambled siRNA.

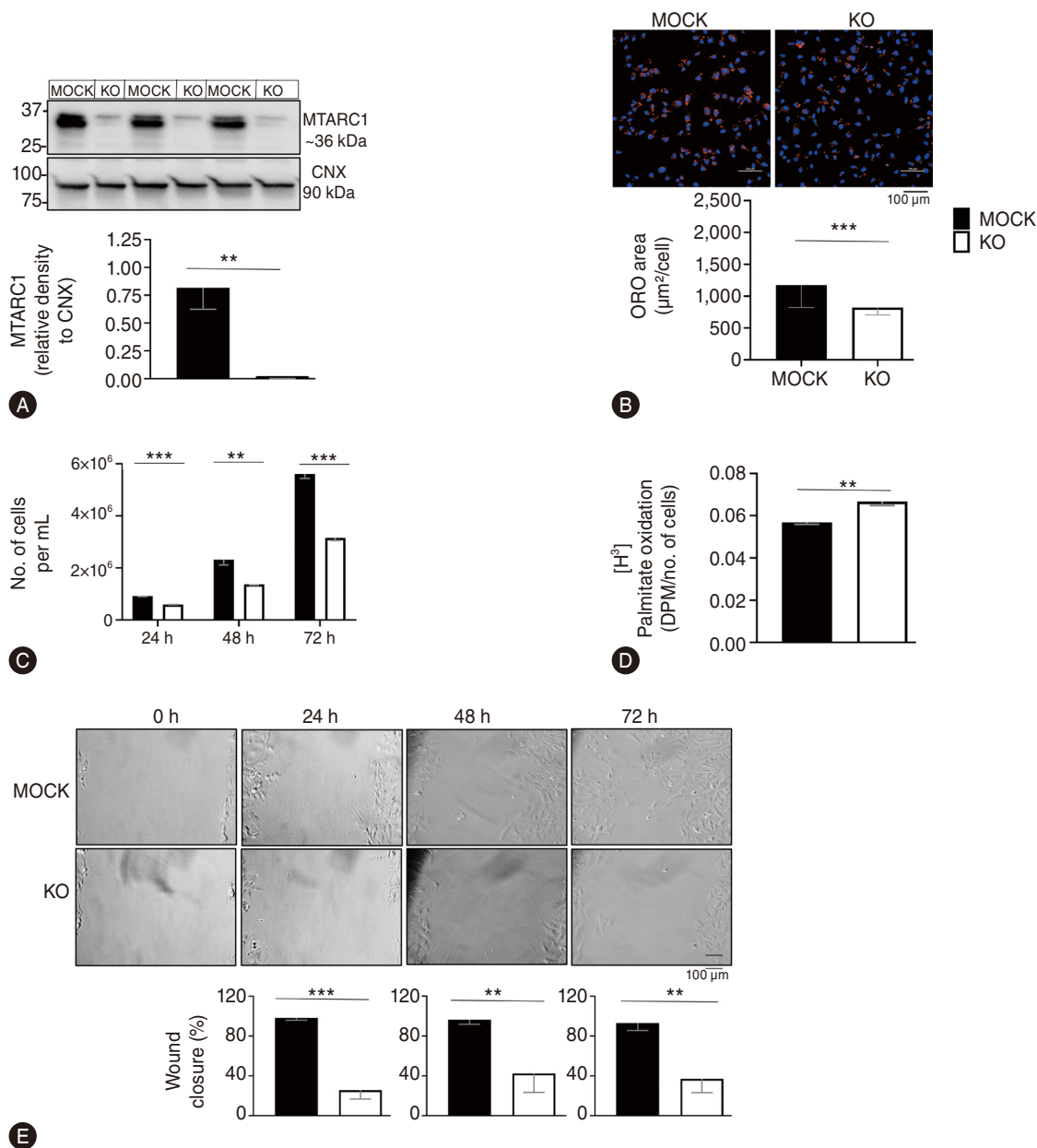


Figure 2. Stable knockout (KO) of *MTARC1* p.A165 in Hep3B2 cells reduces their proliferation, lipid content and migration. (A) Immunoblotting was performed to assess *MTARC1* KO efficiency. Cell lysates were run in SDS-PAGE and probed against *MTARC1* and CNX. Band intensities were normalized to CNX and quantified using ImageLab software. Statistical analysis was performed using unpaired Student's *t*-test. (B) Hep3B2 mock or *MTARC1* p.A165 KO cells were seeded and fixed after 48 hours in 4% PFA, followed by Oil Red O (ORO) and DAPI staining. The ORO stained area was quantified using ImageJ. Objective: 20×, DAPI: Blue, ORO: Red. Scale bar=100 μm. *P*-values were calculated by Mann–Whitney non-parametric test. (C) Hep3B2 mock or *MTARC1* p.A165 KO cells were collected at 24 hours, 48 hours, and 72 hours, and cell counts were performed. *P*-values were calculated using an unpaired Student's *t*-test. (D) Cells were incubated with 8.3 μCi/mL [³H] palmitate and 110 μM palmitate for 2 hours. Media were collected, and radioactivity was measured using a scintillation counter. Results were expressed as disintegrations per minute (DPM) and normalized to cell number. *P*-values were calculated by Mann–Whitney non-parametric test. (E) A scratch wound assay was performed in Hep3B2 mock cells and *MTARC1* KO cells. After 24 hours of seeding, a scratch was made using pipette tips and images were captured at 24 hours, 48 hours, and 72 hours. Objective: 20×. Scale bar=100 μm. Data are normalized to the mock control at each respective time point (set as 100%). Wound closure was calculated in percentage. *P*-values were calculated using an unpaired Student's *t*-test. Data are shown as mean±SD for three independent experiments. Statistical analysis was performed using GraphPad Prism version 9 (San Diego, CA, USA). ***P*<0.01, ****P*<0.001. CNX, calnexin; *MTARC1*, mitochondrial amidoxime-reducing component 1; PFA, Paraformaldehyde.

Fatty acid degradation is a cellular metabolic pathway that involves the breakdown of fatty acids to generate metabolic energy.⁶ By proteomic profiling we found higher levels of proteins involved in fatty acid degradation, including HMGCS1, GPX1, FMO1 and CPT1A, in *MTARC1* p.A165 KO cells compared with mock cells (Fig. 3D).

The TGF- β pathway is known to promote cancer progression, metastasis and extracellular matrix remodeling.¹⁴ We found that the levels of TGFB2 and RRAS were lower, whereas SKI was higher, in *MTARC1* p.A165 KO cells compared with mock cells, indicating suppression of the TGF- β pathway in the KO cells (Fig. 3E).

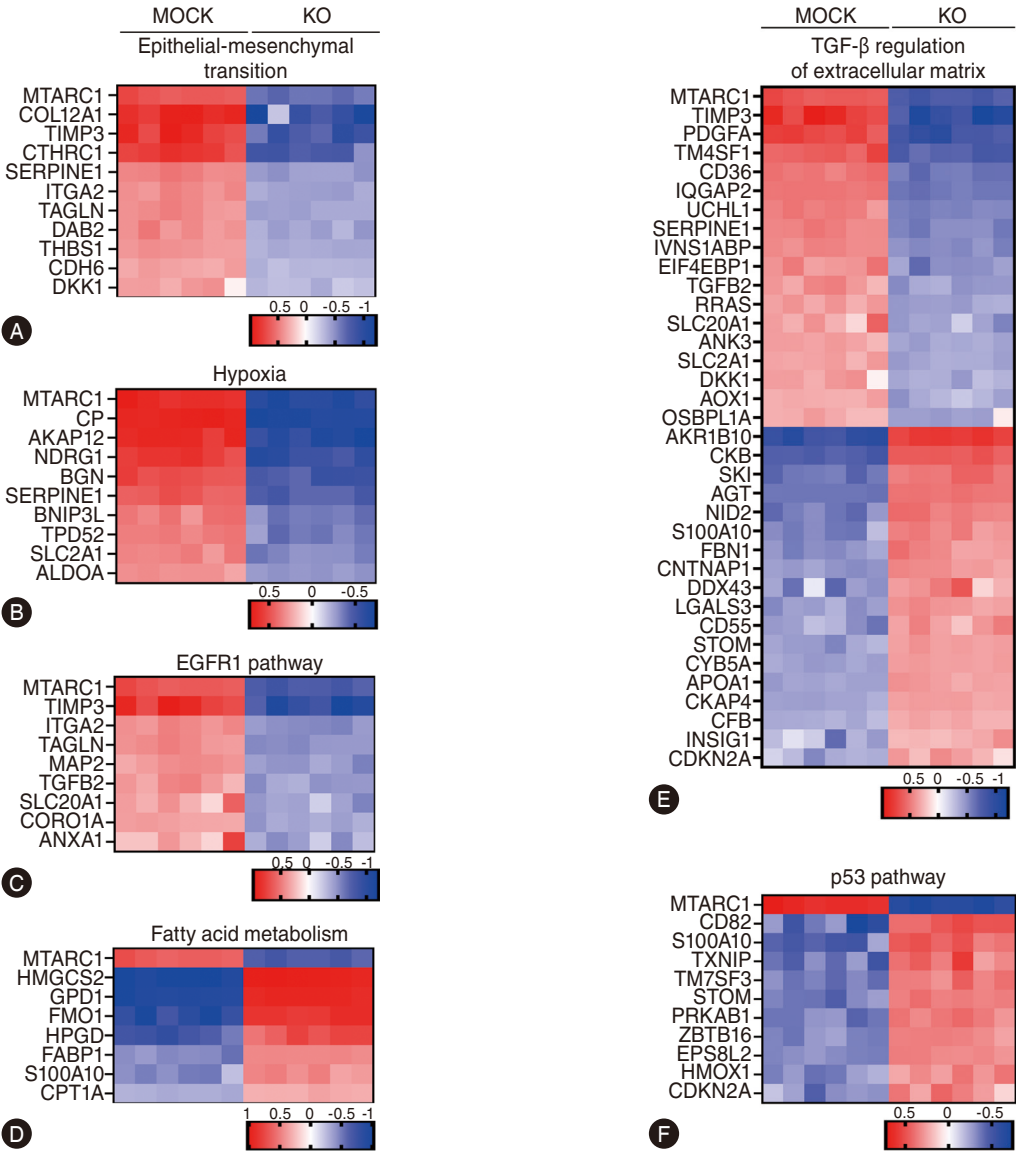


Figure 3. Proteomics analysis of *MTARC1* p.A165 KO in Hep3B2 cells. Whole-cell proteomics was performed on six technical replicates of Hep3B2 mock and *MTARC1* KO cells. Samples were processed and proteins were quantified using MS/MS, liquid chromatography-tandem mass spectrometry. The protein abundance ratios were log2-transformed, and statistical significance was assessed using Benjamini-Hochberg test, with an adjusted *P*-value threshold of <0.05. A $|\log_2FC| \geq \pm 0.58$ cutoff was used to define upregulation or downregulation. The colour intensity in the heatmap represents the Log2FC values. Pathway enrichment analysis was performed using Enrichr, and the following pathways were enriched in *MTARC1* KO cells: (A) EMT, (B) Hypoxia, (C) EGFR1, (D) Fatty acid metabolism, (E) TGF- β regulation of extracellular matrix and (F) p53 pathway. EGFR1, epidermal growth factor receptor 1; EMT, epithelial–mesenchymal transition; KO, knockout; MS/MS, tandem mass spectrometry; *MTARC1*, mitochondrial amidoxime-reducing component 1; TGF- β , transforming growth factor-beta.

Although Hep3B2 cells are null for *TP53* (the gene encoding p53),¹⁵ pathway enrichment analyses highlighted higher p53-related signaling, likely reflecting a p53-independent response converging on similar downstream targets. Notably, the tumor suppressor *CDKN2A* was higher in *MTARC1* p.A165 KO cells compared with mock cells, consistent with the induction of an anti-proliferative pathway (Fig. 3F).

Our findings demonstrate that loss of *MTARC1* p.A165 in Hep3B2 cells leads to suppression of EMT, hypoxia adaptation and EGFR signaling while promoting fatty acid degradation.

We also performed whole-cell proteomics on *MTARC1* p.A165 KO and *MTARC1* mock HepG2 cells. We detected a total of 7,000 proteins, of which 299 were upregulated and 296 were downregulated in the *MTARC1* p.A165 KO cells compared with the mock cells (FDR adjusted, $|\log_2FC| \geq \pm 0.58$). After analysis of the differentially expressed proteins, five proteins were randomly selected whose expression levels were unchanged between HepG2 mock and HepG2 *MTARC1* p.A165 KO cells (Supplementary Fig. 2C). Heatmaps of differentially expressed proteins between mock and *MTARC1* p.A165 KO are shown for each pathway (Supplementary Fig. 3). Loss of *MTARC1* p.A165 in HepG2 cells led to suppression of the mTORC1 pathway, while promoting fatty acid metabolism. Certain EMT markers were increased; however, this should be interpreted cautiously, as TGF- β -associated proteins were decreased (Supplementary Fig. 3), indicating that the observed changes may represent a feedback response rather than bona fide EMT activation.

***CPT1A* knockdown counteracts the beneficial effect of *MTARC1* p.A165 KO on proliferation**

To assess the causal relationship between *MTARC1* p.A165 KO and β -oxidation on proliferation, we transiently knocked down *CPT1A* in Hep3B2 mock and *MTARC1* p.A165 KO cells. In mock cells, si*CPT1A* induced upregulation of *MTARC1* mRNA, suggesting a feedback response to lower fatty acid availability in the mitochondria ($P < 0.01$, Fig. 4A). Consistent with these results, *CPT1A* knockdown lowered β -oxidation by 30% ($P < 0.001$) in mock cells and by 50% ($P < 0.001$) in *MTARC1* p.A165 KO cells compared with their respective siSCR-transfected cells (Fig. 4B).

As a consequence of lower β -oxidation, *CPT1A* knockdown was associated with higher neutral lipid content in both backgrounds. si*CPT1A* knockdown resulted in 59% ($P < 0.001$) higher neutral lipid content compared with mock siSCR. *MTARC1* p.A165 KO siSCR exhibited 42% ($P < 0.001$) lower neutral lipid content than mock siSCR. The double perturbation (*MTARC1* p.A165 KO+si*CPT1A*) showed 41% ($P < 0.001$) higher neutral lipid content relative to *MTARC1* p.A165 KO siSCR but remained 18% ($P < 0.001$) lower than mock siSCR, indicating a partial rescue of the KO phenotype (Fig. 4C).

Similarly, *CPT1A* knockdown enhanced cell proliferation. si*CPT1A* knockdown resulted in a 22% ($P < 0.01$) higher number of cells than mock siSCR. *MTARC1* p.A165 KO siSCR exhibited 49% ($P < 0.01$) lower cell numbers than mock siSCR cells. The double perturbation (*MTARC1* p.A165 KO+si*CPT1A*) showed 65% ($P < 0.01$) higher cell numbers relative to *MTARC1* p.A165 KO siSCR but remained 16% ($P < 0.01$) lower than mock siSCR, again indicating a partial rescue of the KO phenotype (Fig. 4D).

Together, these findings demonstrate that *CPT1A* knockdown reduces β -oxidation and partially reverses, the beneficial effect observed in *MTARC1* p.A165 KO cells, demonstrating a causal role for β -oxidation in this phenotype.

KO of *MTARC1* p.A165 reduces tumor growth in a subcutaneous xenograft mouse model

To determine whether *MTARC1* p.A165 reduction influences tumorigenicity in vivo, *MTARC1* p.A165 KO and Hep3B2 mock cells were injected subcutaneously into the flanks of 5-week-old BALB/c nude mice (Supplementary Fig. 4). Tumor growth was monitored after injection over a period of 48 days, with tumor volumes measured every 3 days (Supplementary Fig. 4).

Tumors derived from mock cells grew more rapidly, whereas those from *MTARC1* p.A165 KO cells showed markedly lower growth (Fig. 5A, top and middle panels). At the end point, gross examination revealed smaller tumors in the *MTARC1* p.A165 KO group, which also appeared less red and less vascularized compared with the mock tumors (Fig. 5A, bottom panel).

Longitudinal measurements of tumor volume revealed a sustained difference between groups throughout the study, with a lower volume observed from day 36 after injection

onwards, until the end of the experiment (day 48). By day 48, tumor volume in the *MTARC1* p.A165 KO group was 45% lower compared to the mock controls, indicating a reduction in tumor burden among the KO group (Fig. 5B; $P<0.001$). Consistent with these results, tumor weight was also 38% lower in the *MTARC1* p.A165 KO group compared to the mock group (Fig. 4C; $P<0.05$). Additionally, we found that the expression of Ki-67, a marker of cell proliferation, was 42% lower in the *MTARC1* p.A165 KO tumors compared with mock tumors, as visualized by immunofluorescence (Fig. 5C; $P<0.05$).

These results demonstrate that *MTARC1* p.A165 KO in-

hibits tumor growth in a subcutaneous xenograft mouse model.

In vivo tumor proteomics reveals that *MTARC1* p.A165 KO downregulates oncogenic pathways in HCC

To investigate the molecular consequences of *MTARC1* p.A165 KO in Hep3B2 xenograft tumors, we performed whole-cell proteomic analysis comparing protein expression profiles between Hep3B2 mock and Hep3B2 *MTARC1* p.A165 KO xenografts in nude mice. We detected 8,381

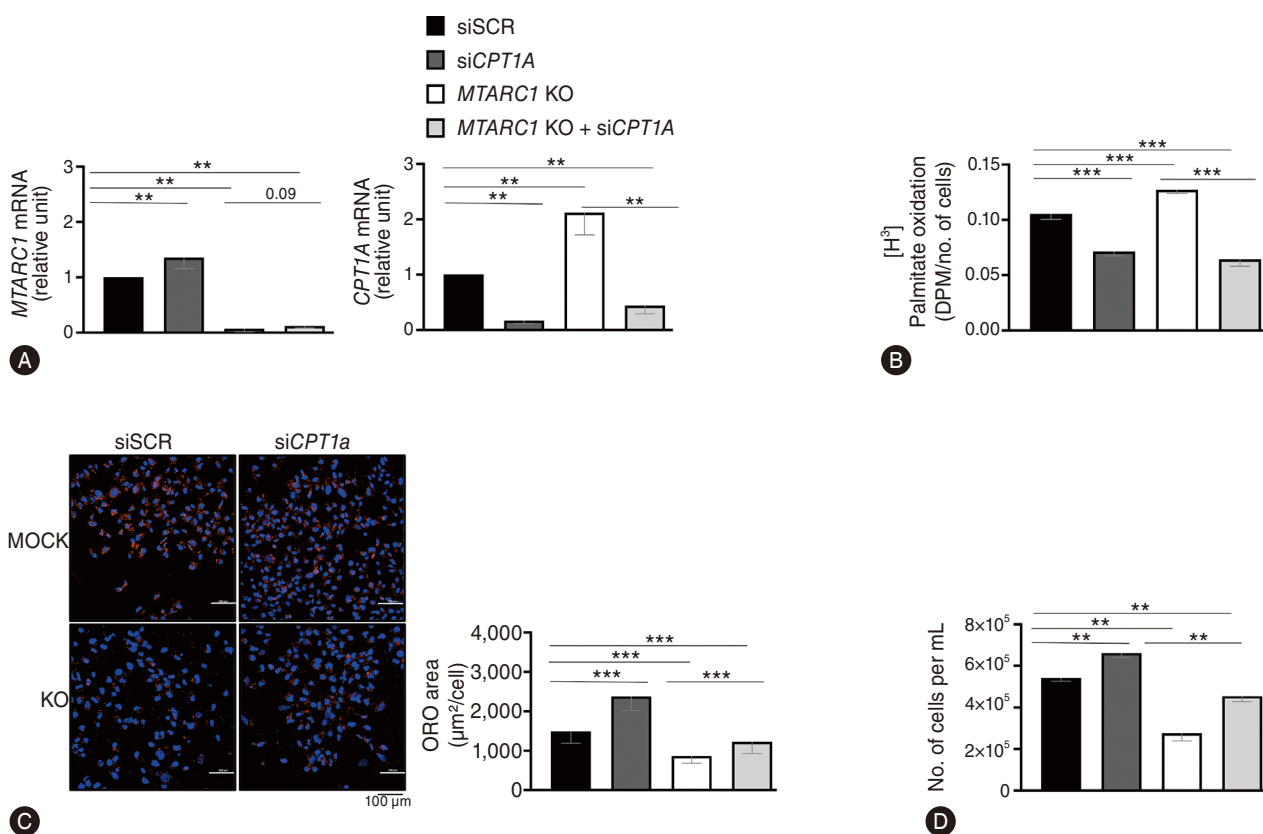


Figure 4. *CPT1A* knockdown reduces β -oxidation and partially rescues the phenotype observed in *MTARC1* p.A165 knockout (KO) cells. Hep3B2 mock or *MTARC1* p.A165 KO were seeded and, after 24 hours, transfected with scrambled siRNA (siSCR) or *CPT1A* targeting siRNA (siCPT1A). Cells were subsequently collected for the indicated analyses. (A) Relative mRNA expression of *MTARC1* (left) and *CPT1A* (right) determined by qRT-PCR in mock and *MTARC1* p.A165 KO following siSCR or siCPT1A transfection. Data are expressed as relative units (RU). (B) β -oxidation measured by incubation with 8.3 μ Ci/mL [3 H]-palmitate and 110 μ M palmitate for 2 hours. Media were collected and the radioactivity was quantified using a scintillation counter. Results are expressed as disintegrations per minute (DPM) normalized to cell number. (C) Cells were fixed 48 hours post-transfection with 4% PFA and stained with Oil Red O (ORO) and DAPI. Lipid accumulation was quantified as ORO-positive area per cell using ImageJ. Representative images are shown (objective 20 $\times$; DAPI, blue; ORO, red). Scale bar=100 μ m. (D) Cell counts were performed 48 hours post-transfection. Data are shown as mean $\pm$ SD from six independent experiments. Statistical significance was assessed using the Mann–Whitney non-parametric test and analyzed with GraphPad Prism version 9 (San Diego, CA, USA). ** $P<0.01$, and *** $P<0.001$. *MTARC1*, mitochondrial amidoxime-reducing component 1; PFA, Paraformaldehyde.

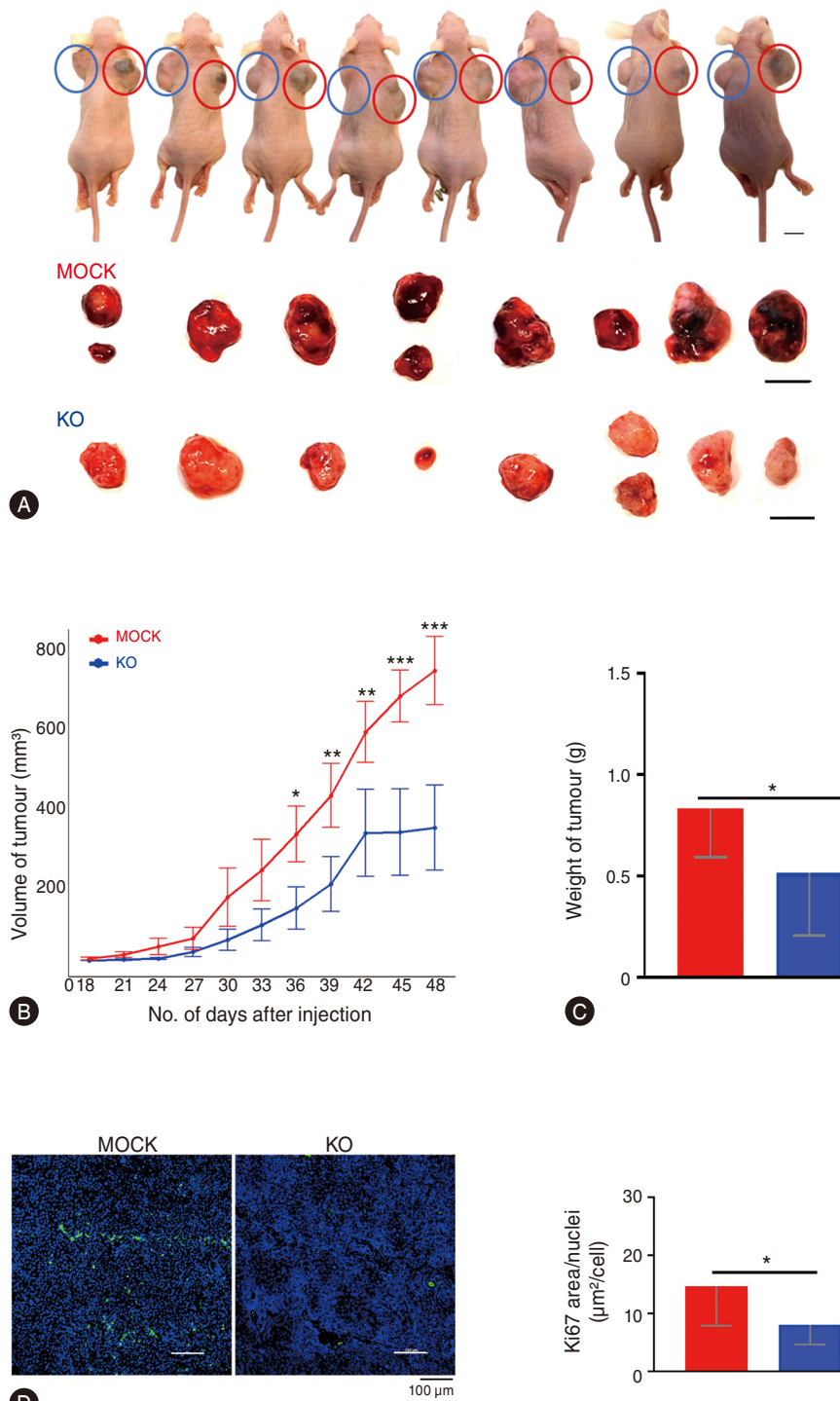


Figure 5. *MTARC1* p.A165 knockout (KO) reduces tumor volume and weight in a subcutaneous xenograft mouse model. (A) Representative images of mice (top) and xenograft tumors (bottom) at the day of termination. Scale bar for mouse: 1.8 mm and scale bar for tumor: 0.38 mm. (B) Tumor volume was measured every 3 days. Statistical significance was determined using a linear mixed model. Linear mixed-effects model was fitted using R. (C) Tumor weight was measured on the day of termination. The *P*-value was calculated by Mann–Whitney non-parametric test. (D) Representative tumor sections were processed for immunofluorescence with an anti-Ki-67 antibody, and the nuclei were stained with DAPI (blue). Objective: 20×, DAPI: Blue, Ki-67: Green. Scale bar=100 μm. *P*-values were calculated by Mann–Whitney non-parametric test. Statistical analysis was performed using GraphPad Prism version 9 (San Diego, CA, USA). **P*<0.05, ***P*<0.01, and ****P*<0.001. *MTARC1*, mitochondrial amidoxime-reducing component 1.

proteins, of which 375 were upregulated and 228 were downregulated in Hep3B2 *MTARC1* p.A165 KO xenografts compared with the mock control group (FDR adjusted, $|\log_2\text{FC}| \geq \pm 0.58$).

In Hep3B2 2D proteomics (mock vs. *MTARC1* p.A165 KO), we detected 428 differentially expressed proteins, whereas in xenografted tumors in nude mice (mock vs. *MTARC1* p. A165 KO), we detected 603 differentially expressed proteins. Notably, 111 proteins were commonly differentially expressed across both models. Among these shared proteins, 102 proteins exhibited concordant expression changes while 9 displayed discordant regulation. The inverse regulation may reflect adaptation of tumor cells to the in vivo microenvironment and contributions from host-tumor interactions within the xenograft setting.

The overall expression patterns demonstrated a strong positive correlation in protein expression between Hep3B2 mock and *MTARC1* p.A165 KO cells and their corresponding xenografted tumors, with a Log_2FC concordance yielding a Pearson correlation coefficient of $r=0.76$ ($P=5.7 \times 10^{-22}$) (Fig. 6A).

Pathway enrichment analysis revealed changes in erythrocytic proteins, hypoxia signaling, myc repressed pathway, G2-M checkpoint and p53 signaling. Heatmaps of differentially expressed proteins between mock tumors and KO tumors are shown in each pathway (Fig. 6). After analysis of the differentially expressed proteins, five proteins were randomly selected whose expression levels did not differ between Hep3B2 mock and Hep3B2 *MTARC1* p.A165 KO xenograft tumors (Supplementary Fig. 2B).

We observed lower expression of several erythrocytic markers, including HBA1, HBB and SPTB, in *MTARC1* p.A165 KO tumors compared with mock tumors (Fig. 6B).

Hypoxia pathway analysis revealed lower expression of hypoxia-related proteins, including PGAM2, EFNA1 and GALK1, in *MTARC1* p.A165 KO tumors compared with mock tumors. This indicates that loss of *MTARC1* reduces hypoxic stress within tumors. The combined effect of reduced erythrocytic and hypoxia markers likely contributes to the pale appearance of *MTARC1* p.A165 KO tumors, reflecting lower vascularization compared with mock tumors (Fig. 6C).

Despite the fact that Hep3B2 cells are *TP53* null,¹⁵ *MTARC1* KO exhibited higher levels of p53-associated proteins, including PRKAB1, HEXIM1, and ZFP36L1. These

proteins act as tumor suppressors in various cancers, and their increased expression suggests that *MTARC1* loss can restrict tumor growth even in the absence of p53 (Fig. 6D).

We also found increases in several G2/M checkpoints proteins in *MTARC1* p.A165 KO tumors compared with mock tumors, which can lead to cell cycle arrest in response to stress. Notably, CDKN1B was higher in the KO tumors, potentially blocking cyclin–CDK activity and ceasing their progression into mitosis (Fig. 6E).

Notably, in *MTARC1* p.A165 KO tumors we observed an increase in the proteins that inhibit the Myc pathway, such as CDKN1B, FOXO3, and SMAD2, suggesting a role for *MTARC1* in this pathway (Fig. 6F).

DISCUSSION

The main finding of this study is that *MARC1* p.A165 ablation reduces HCC aggressiveness by metabolic reprogramming.

We began by assessing proliferation in human HCC cell lines homozygous for the *MTARC1* risk allele (p.A165). Transient siRNA-mediated knockdown of *MTARC1* p.A165 led to lower proliferation in four HCC cell lines (Hep3B2, HuH7, HepG2 and HepaRG). These results were validated in CRISPR-Cas9-mediated stable *MTARC1* p.A165 KO Hep3B2 cells, in which we found a larger effect size compared to the transient knockdown approach, likely reflecting the stable nature of CRISPR-Cas9-mediated KO. In addition to the effects on proliferation and migration, Hep3B2 *MTARC1* p.A165 KO displayed reduced intracellular neutral lipid content and increased fatty acid β -oxidation compared to mock KO control cells, consistent with our previous work with human primary hepatocytes.⁷ We should note that transient knockdown of *CPT1A* partially reversed the beneficial effect of the *MTARC1* p.A165 KO, indicating that the increase in β -oxidation induced by the KO underlies this beneficial effect.

To further explore the molecular mechanisms underlying the role of *MTARC1* in HCC, we performed proteomic profiling in vitro using Hep3B2 mock and *MTARC1* KO cells. Loss of *MTARC1* p.A165 led to suppression of key oncogenic pathways, including EMT, EGFR signaling, TGF- β -mediated extracellular matrix remodeling and hypoxia responses. Downregulation of EMT markers (CTHRC1,

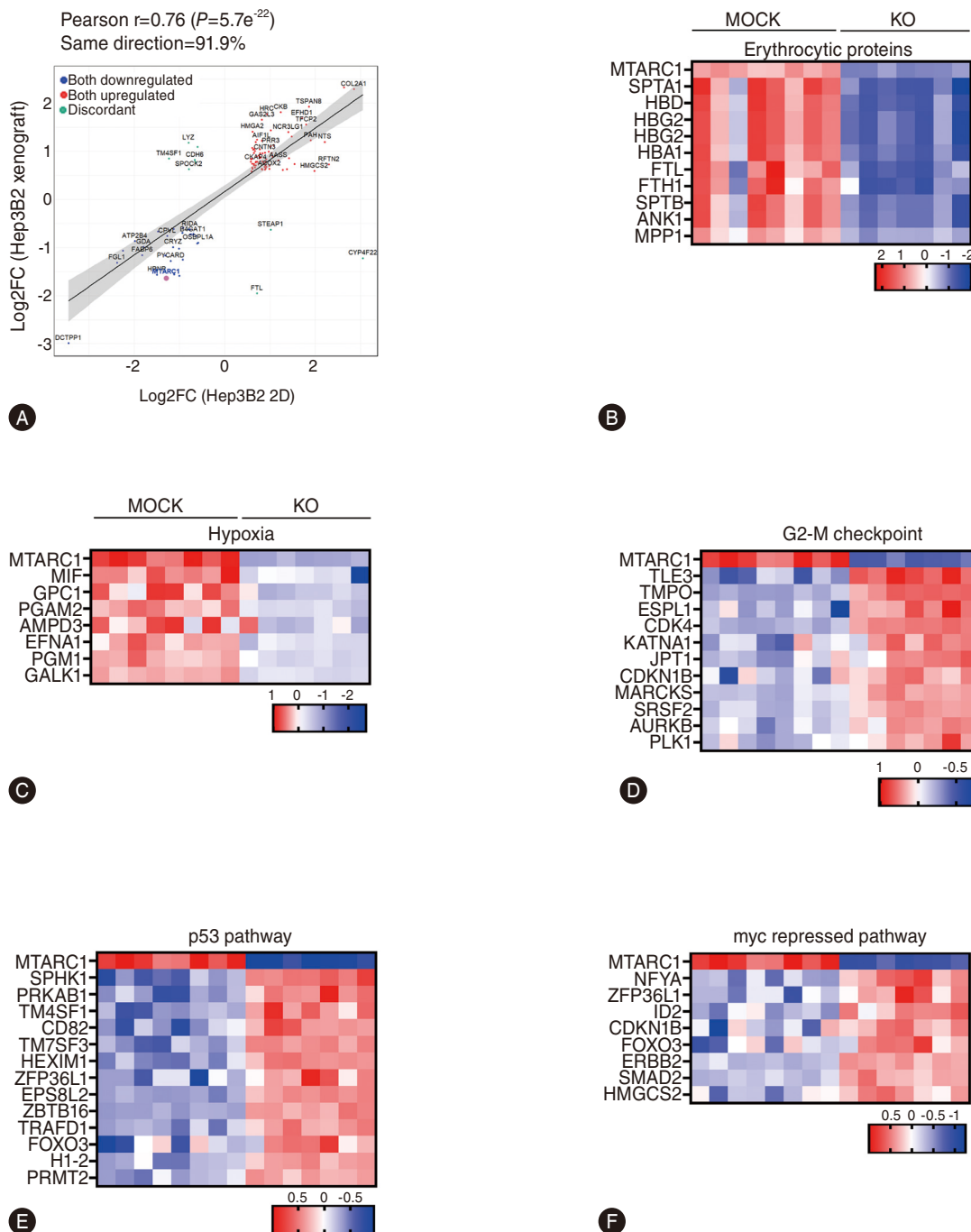


Figure 6. Proteomic profiling of xenografted tumors reveals downregulation of oncogenic pathways in *MTARC1* p.A165 knockout (KO) tumors. Whole-cell proteomics was performed on 8 Hep3B2 mock and 7 *MTARC1* p.A165 KO xenografted tumors. Samples were processed and proteins were quantified using MS/MS, liquid chromatography-tandem mass spectrometry. The protein abundance ratios were log₂-transformed, and statistical significance was assessed using Benjamini-Hochberg test, with an adjusted *P*-value threshold of <0.05. A $|\log_2FC| \geq 0.58$ cutoff was used to define upregulation or downregulation. (A) Correlation of log₂FC in protein expression between Hep3B2 mock and *MTARC1* KO cells and their corresponding xenografted tumors in nude mice. Red colour indicates upregulated proteins while blue colour indicates downregulated proteins in both cell lines and tumors. The correlation highlights the similarities and differences in protein expression profiles between in vitro and in vivo settings. Pearson's correlation coefficient was calculated in R. The colour intensity in the heatmap represents the Log₂FC values. Pathway enrichment analysis was performed using Enrichr, and the following pathways were enriched in *MTARC1* KO tumors: (B) Erythrocytic proteins, (C) Hypoxia, (D) p53 pathway, (E) G2-M checkpoint, and (F) Myc repressed pathway. *MTARC1*, mitochondrial amidoxime-reducing component 1; MS/MS, tandem mass spectrometry.

THBS1) and hypoxia-related proteins (NDRG1, ALDOA) indicates impaired cellular plasticity and adaptation to hypoxic stress, both of which are critical for HCC aggressiveness.¹⁶⁻¹⁹ Interestingly, we found that *MTARC1* p.A165 KO increased levels of tumor suppressor-associated proteins (CDKN2A, ZBTB16), suggesting activation of anti-proliferative pathways, even in Hep3B2 cells, which are p53 deficient. All together, these findings indicate that *MTARC1* contributes to tumor progression by orchestrating oncogenic signaling cascades. In vivo, we found that *MTARC1* p.A165 KO had profound effects on tumor growth. Xenograft tumors derived from *MTARC1* p.A165 KO Hep3B2 cells exhibited slower growth, reduced vascularization and smaller size compared with Hep3B2 mock cells. We also found a 42% reduction in Ki-67 expression, a well-known proliferation marker upregulated in HCC,²⁰ in *MTARC1* p.A165 KO tumors. Consistent with these observations, erythrocyte markers and hypoxia-related proteins were lower in *MTARC1* p.A165 KO tumors, supporting the notion that *MTARC1* influences both tumor metabolism and its microenvironment. Interestingly, by proteomics, we found 30% more differentially expressed proteins in xenograft tumors compared with 2D cell culture, highlighting the complexity of host-tumor interactions and involvement of additional signaling pathways in vivo.

The missense variant p.A165T (rs2642438) is associated with a small protective effect on HCC risk with an odds ratio (OR) of ~0.83, which is attenuated when HCC cases are compared specifically with cirrhosis without HCC (OR 0.91; 95% CI 0.84–1.00), indicating that the observed effect of the variant is shared with progression to cirrhosis rather than tumor development per se.¹⁰

In contrast, a recent genome-wide meta-analysis identified a separate *MTARC1* signal, an intronic variant (rs2642442), which reaches genome-wide significance and is associated with a modest increase in risk (β ~0.17, corresponding to an OR of ~1.2).²¹ The genetic effect correlates strongly with genetic determinants of hepatic steatosis ($r^2=0.75$) and cirrhosis ($r^2=0.69$). These data support a model in which this variant influences HCC risk primarily through hepatic lipid accumulation and progression of chronic liver disease. Notably, this intronic variant is compatible with a gain-of-function and is associated with an increased risk of MASLD (OR of 1.15).²²

Overall, both studies are consistent with typical common

germline variants, supporting a role for *MTARC1* as a modifier of risk rather than a strong oncogenic driver. Also, our study focuses on germline variation and metabolic regulation rather than somatic mutagenesis.

The modest effect size associated with the *MTARC1* p.A165 variant in humans contrasts with the much larger effects observed following *MTARC1* KO in cancer cell lines. There are at least three plausible explanations for the observed discrepancy: (1) in our hands, the *MTARC1* p.A165 variant reduces protein levels by approximately 50%,^{23,24} whereas CRISPR-mediated KO virtually eliminates the protein, resulting in a stronger perturbation of the system; (2) people born with the *MTARC1* protective genetic variant may develop compensatory mechanisms as a consequence of lifelong reduction in *MTARC1* function, whereas acute ablation of *MTARC1* on a wild-type background would not allow for such adaptation and may therefore produce a larger effect size. An illustrative example is congenital analbuminemia, a largely benign condition characterized by oedematous swelling, in contrast to acquired severe hypoalbuminemia, which is a life-threatening condition²⁵; and (3) in carriers of the genetic variant, *MTARC1* loss of function occurs at the whole-body level (including the immune system), whereas our experiments are limited to hepatocytes.

Our study has some limitations. All experiments were conducted in HCC cells carrying the *MTARC1* risk variant (p.A165) and not in cells expressing the protective p.T165 allele. We have previously demonstrated that the protective allele leads to a loss of *MTARC1* function through proteasomal degradation.²³ Based on these findings, silencing the protective allele would be expected to have little or no impact on cell proliferation and metabolic reprogramming. Moreover, our in vivo studies were performed in nude mice, which do not reflect the interaction with the immune system. Further studies in orthotopic models or humanized mouse systems will be valuable to confirm these results. Finally, our proteomic analyses revealed suppression of various oncogenic pathways. The causal relationship between *MTARC1* ablation and these signaling cascades remains to be defined.

In conclusion, our results demonstrate that *MTARC1* p.A165 ablation results in metabolic reprogramming attenuating HCC aggressiveness by downregulating oncogenic and upregulating tumor suppressor signals. These data

suggest that downregulation of MTARC1 p.A165 may be a potential strategy to treat HCC.

Authors' Contribution

LK contributed to study design, data collection, data analysis, data interpretation, and manuscript drafting; JZ and FGM were involved in data collection and visualization; TD was involved in data interpretation and manuscript drafting; XG and BA were involved in data collection and visualization; OJ was involved in data interpretation; PI and EB were involved in visualization and manuscript drafting; MM was involved in data collection and visualization; RMM contributed to study design, supervision, discussion, and manuscript writing; SR led the study with conceptualization, funding acquisition, supervision, data interpretation, methodology, and manuscript writing. All authors read and approved the final version for publication.

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Conflicts of Interest

In the last 5 years S.R. received research grants from Novonordisk and AstraZeneca for basic science research on steatotic liver disease, and has been consulting for As-

traZeneca, GSK, Celgene Corporation, Ribocure AB, Madrigal, Ultragenyx, Amgen, Sanofi, Wave Life Sciences, Lipigon, Novartis, Profluent, Aina, Echosense and Chiesi in the last 5 years; declares equity from Heptabio; and is inventor on a Patent with title "Method for treating fatty liver disease", on PSD3, US application number 17,480266 filed on 21st September 2021. All other authors have none to declare.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (<http://www.e-cmh.org>).

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