GLUCOSE DEPRIVATION INDUCED ABERRANT FUT1-MEDIATED FUCOSYLATION DRIVES CANCER STEMNESS IN HEPATOCELLULAR CARCINOMA

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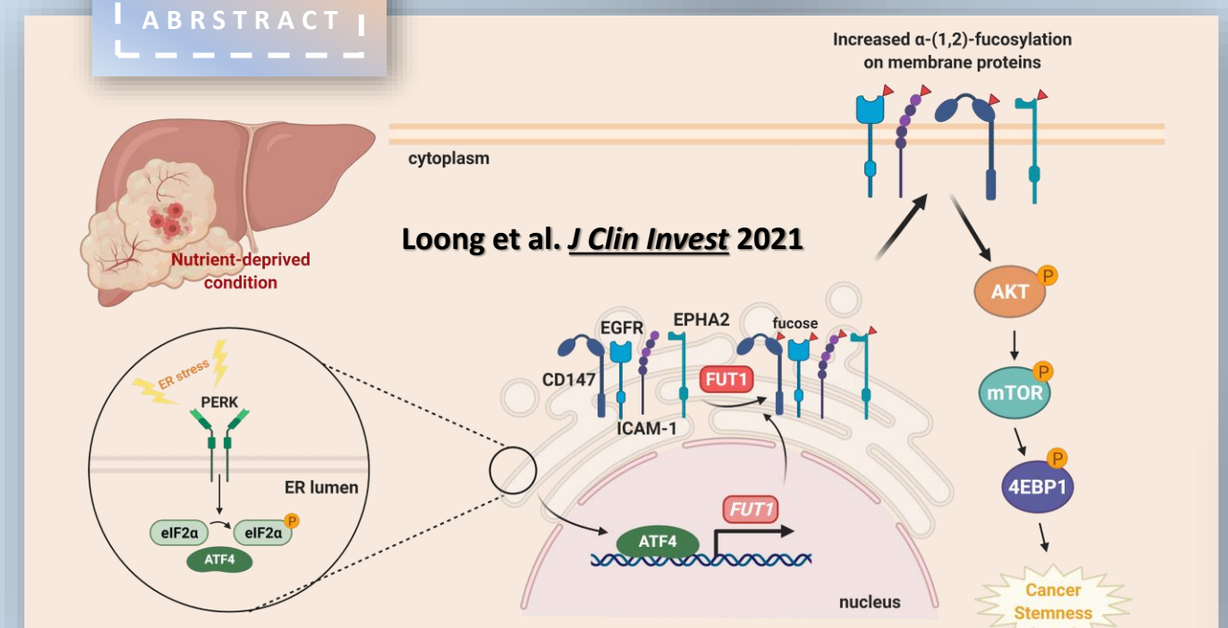
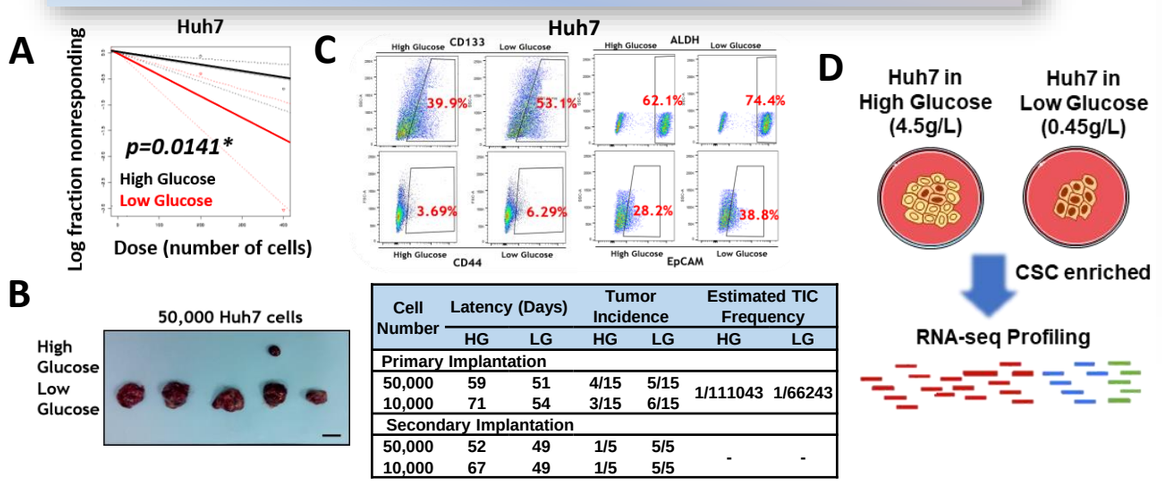
GRAPHICAL ABRSTRACT



INTRODUCTION

Tumor cells often respond to the cytotoxic effects of metabolic stresses by inducing molecular adaptations that promote survival rather than a proliferative response, resulting in clonal selection of a more malignant phenotype. The core of the bulk tumor is believed to be poorly vascularized, with glucose levels decreasing from the tumor periphery to the interior. We found that low glucose availability, commonly observed in the microenvironment of large growing tumors such as hepatocellular carcinoma (HCC), could enrich for a tumor-initiating cell (TIC) phenotype and enhance PERK-mediated ATF4 expression to drive FUT1 expression, which work hand-in-hand to promote tumor initiation and drug resistance.

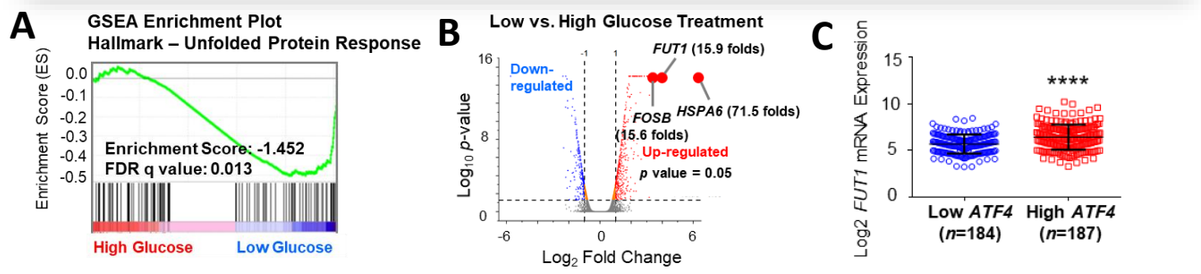
1. GLUCOSE DEPRIVATION RESULTS IN ENHANCED CANCER STEMNESS PROPERTIES



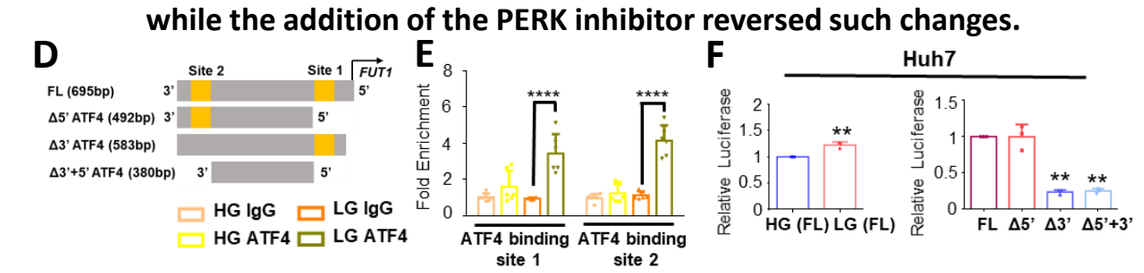
Cell Number	Latency (Days)		Tumor Incidence		Estimated TIC Frequency	
	HG	LG	HG	LG	HG	LG
Primary Implantation						
50,000	59	51	4/15	5/15	1/111043	1/66243
10,000	71	54	3/15	6/15		
Secondary Implantation						
50,000	52	49	1/5	5/5	-	-
10,000	67	49	1/5	5/5		

Figure 1. (A) *In vitro* limiting dilution assay of high/low glucose treated Huh7 HCC cells. (B) *In vivo* limiting dilution and serial transplantation assay in high/low glucose treated Huh7 cells. (C) CD133, ALDH, CD44 and EpCAM protein levels in high/low glucose treated cells. (D) Strategy for mRNA profiling to identify altered transcriptomes of HCC cells grown in high or low glucose conditions. *p=0.01 to 0.05

2. PERK-MEDIATED UNFOLDED PROTEIN RESPONSE ACTIVATES FUT1 PROMOTER ACTIVITY VIA ATF4 INDUCTION UNDER GLUCOSE RESTRICTION



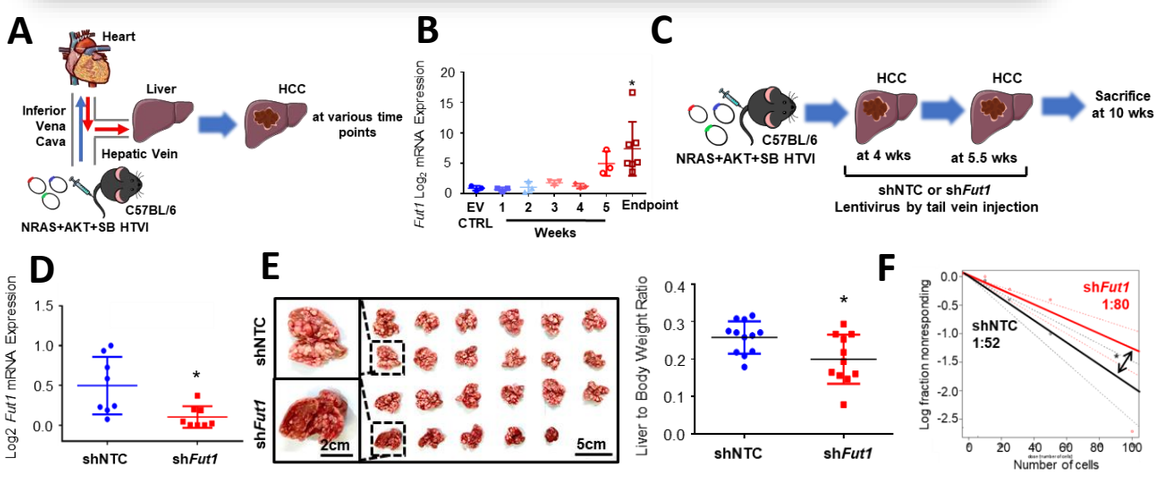
Increased protein levels of unfolded protein response key molecular players - GRP78, p-PERK, p-eIF2α, ATF4 are observed in HCC cells under glucose restriction, while the addition of the PERK inhibitor reversed such changes.



Both mRNA and protein expressions of FUT1 are found to be overexpressed in HCC clinically. FUT1 overexpression also tightly associated with aggressive clinical features.

Figure 2. (A) Gene set enrichment analysis (GSEA) of differentially expressed genes identified by RNA-seq found that the PERK-mediated unfolded protein response was highly enriched in HCC cells cultured under low glucose conditions. (B) A volcano plot revealed the top three most significantly enhanced genes when HCC cells were cultured in low glucose. (C) Analysis of FUT1 and ATF4 expression in The Cancer Genome Atlas (TCGA) – Liver Hepatocellular Carcinoma (LIHC) database. (D) Schematic diagram illustration of full-length (FL) and deletion mutants of FUT1 used in this study. (E) Confirmation of ATF4 binding to both predicted sites on FUT1 by ChIP-qPCR using ATF4 and IgG antibodies. (F) Luciferase reporter assays found that only the ATF4 binding site 2 (Δ3') was critical in modulating FUT1 transcriptional activity and FUT1 transcriptional activity was upregulated under glucose-deprived conditions in Huh7 cells. ** and **** indicate p<0.01 and p<0.0001, respectively.

3. FUT1 KNOCKDOWN ATTENUATES HEPATOCARCINOGENESIS *IN VIVO*



***Enhanced ability to resist to sorafenib treatment* and *enriched stem cell frequencies* were observed in FUT1 overexpressing HCC cells, while FUT1 suppression completely reversed such functional phenomena *in vitro*.**

Figure 3. (A) Schematic representation of the hydrodynamic tail vein injection (HTVI) model in C57BL/6 mice. (B) qPCR analysis of Fut1 expression in mice that received HTVI of either empty vector (EV) control or NRAS, AKT and sleeping beauty (SB) transposase for HCC induction with samples collected at various time points. n = 22 in total. (C) Strategy for testing the functional significance of Fut1 in hepatocarcinogenesis. (D) Successful Fut1 knockdown confirmed by qPCR. n = 11-12 per group. (E) Representative images of dissected livers at the end of the experiment. Scale bar = 5 cm. Scale bar of representative enlarged tumors at 2 cm. (F) An *ex vivo* limiting dilution assay of HCC tumor cells harvested from HTVI mouse models found that the frequency of tumor-initiating cells decreased in the mice administered shFut1 lentiviral particles. *p=0.01 to 0.05

4. INHIBITION OF α-(1,2) FUCOSYLATION BY 2DGAL INCREASES THE EFFICACY OF SORAFENIB AND ERADICATES TUMOR-INITIATING CELLS

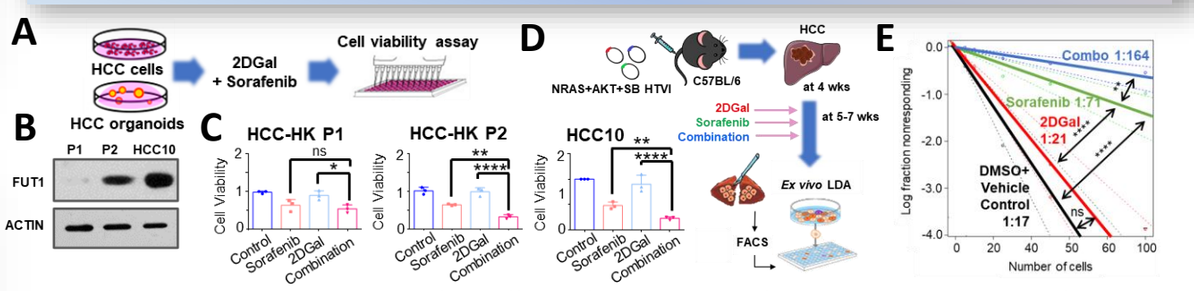


Figure 4. (A) Schematic representation of the effects of 2DGal and sorafenib on Huh7 HCC cell proliferation and HCC patient-derived organoids. (B) Western blot analysis of FUT1 expression in HCC patient-derived organoids HK P1, HCC-HK P2 and HCC10. (C) CellTiter Glo analysis found that FUT1-expressing HCC organoids (D) Strategy for testing the effects of 2DGal and sorafenib in NRAS/AKT/SB HTVI-driven HCC immunocompetent mouse models. (E) *Ex vivo* limiting dilution assay of HCC tumor cells harvested from the HTVI mouse models. *, ** and **** indicate p<0.05, p<0.01 and p<0.0001, respectively.

5. CD147, ICAM-1, EGFR AND EPHA2 ARE FUT1 MEDIATORS THAT REGULATE CANCER STEMNESS THROUGH THE AKT-MTOR-4EBP1 SIGNALING AXIS

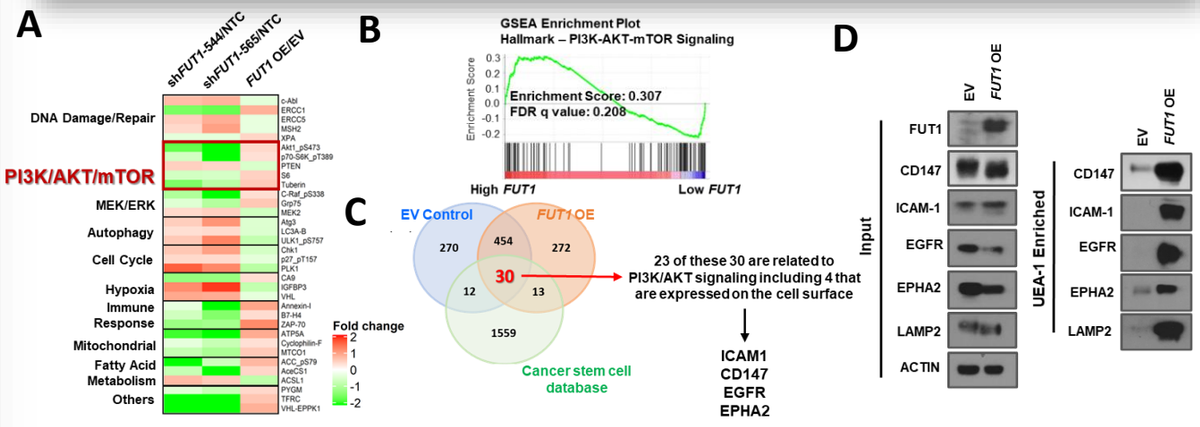


Figure 5. (A) A reversed-phase protein array was performed using Huh7 HCC cell lysates transfected with shNTC (non-target control) or shFUT1 (clones 544 and 565) or transfected with empty vector (EV) control or FUT1 overexpression (FUT1 OE) vector. A heatmap of differentially expressed proteins is shown. (B) GSEA of high FUT1-expressing HCC (top 50%) and low FUT1-expressing HCC (bottom 50%) with data extracted from TCGA LIHC. (C) Venn diagram showing the number of proteins identified by nano LC MS/MS analysis of the UEA-1-enriched fractions of both EV control and FUT1 OE cells, as well as the common proteins that matched the Cancer Stem Cell database (CSCdb). (D) UEA-1 affinity chromatography of whole cell lysates of Huh7 HCC cells transfected with EV or with FUT1 overexpressed.

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