

The Role of Metabolic Enzyme PFKFB3 in Mediating Matrix Stiffness-Induced Tumor Growth and Radiotherapy Resistance in HCC

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Abstract

While the impact of increased matrix stiffness on promoting aggressive behaviors in hepatocellular carcinoma (HCC) cells is well established, its influence on chemoradiotherapy resistance and the precise mechanisms underlying this effect remain unclear. To investigate the contribution of matrix stiffness to HCC development, we developed innovative in vivo animal models featuring precisely controlled liver stiffness levels, along with a matching adjustable hydrogel-based culture platform. This combined strategy enabled detailed analysis of how mechanical signals affect HCC cell growth and DNA repair processes in both laboratory and living-organism settings. Elevated stiffness markedly boosted cell proliferation and protected cells against DNA damage by altering the expression and localization of the metabolic enzyme PFKFB3. In particular, high stiffness reduced PFKFB3 ubiquitination by decreasing E3 ubiquitin ligase NEDD4 levels, thereby stabilizing PFKFB3 protein levels. This led to enhanced glycolysis and ultimately supported HCC expansion. Additionally, stiff matrix conditions improved the DNA damage repair capacity of irradiated HCC cells, with PFKFB3 nuclear translocation playing a key role in stiffness-dependent repair through its interaction with Ku70. These findings reveal a PFKFB3-dependent mechanism by which heightened matrix stiffness drives HCC progression and reduces radiotherapy effectiveness. The study underscores the importance of matrix stiffness in tumor expansion and DNA repair in HCC, while uncovering a novel non-metabolic role for PFKFB3.

Keywords: Hepatocellular carcinoma (HCC), Matrix stiffness, PFKFB3, Radiotherapy resistance, DNA damage repair, Glycolysis

Introduction

Resistance to chemoradiotherapy represents a significant obstacle in managing advanced solid tumors [1, 2]. DNA damage is a primary target of radiotherapy and many chemotherapeutic agents against cancer cells, with DNA

repair proficiency largely dictating treatment outcomes and tumor sensitivity [3, 4]. Therefore, identifying strategies to impair DNA repair mechanisms holds substantial promise for overcoming this therapeutic barrier. Beyond tumor-intrinsic elements—such as genetic alterations, metabolic shifts, oxidative stress, dysregulated cell cycle control, apoptosis evasion, DNA repair proficiency, and cancer stem cell properties [5-8]—external microenvironmental cues, including physical and chemical signals as well as interactions with non-cancerous cells, also shape tumor responses to chemoradiotherapy [9-11]. For instance, low oxygen levels elevate vascular endothelial growth factor expression, thereby enhancing tumor cell survival and

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radiation resistance [12]. Likewise, lactic acid buildup has been shown to promote resistance to both radiotherapy and chemotherapy [13, 14].

Furthermore, an intensified immunosuppressive environment driven by myeloid-derived suppressor cell infiltration further diminishes radiotherapy effectiveness [15, 16]. Such observations underscore the critical role of biochemical factors in DNA repair and treatment sensitivity. Nevertheless, the role of biomechanical cues within the tumor microenvironment in modulating chemoradiotherapy resistance and DNA damage repair has received comparatively little attention.

Mounting data confirm that matrix stiffness, a key biomechanical property of solid tumors, strongly exacerbates malignant traits in cancer cells—including proliferation, motility, invasiveness, and metastatic potential—and accelerates tumor advancement [17-20]. Emerging work also suggests that matrix stiffness can modulate responses to chemoradiotherapy. Limited studies indicate that rigid matrices increase the resistance of ovarian cancer cells to platinum-based DNA-damaging agents [21]. In contrast, matrix stiffening mediated by CNN1hi cancer-associated fibroblasts contributes to 5-fluorouracil resistance in gastric cancer [22]. In HCC, matrix rigidity similarly appears to diminish sensitivity to cytotoxic chemotherapy [23]. Despite these initial links between tissue mechanics and drug resistance, the specific molecular pathways linking matrix stiffness to DNA repair efficiency remain unclear. Uncontrolled proliferation is a defining characteristic of cancer cells. Aerobic glycolysis supplies most of the energy required for this rapid expansion [24]. Levels and activity of key glycolytic enzymes typically mirror the glycolytic state of tumor cells [25, 26]. Beyond their conventional enzymatic roles, several metabolic enzymes exert non-metabolic effects by influencing cellular processes, survival, and disease development through nuclear shuttling [27]. Given the potential of matrix stiffness to affect treatment resistance and the known role of glycolytic enzymes in proliferation and

cell survival, we hypothesized that mechanical stiffness could alter the abundance and subcellular distribution of glycolytic enzymes, thereby promoting HCC growth and radiotherapy resistance.

In this investigation, we created unique animal models with tunable liver stiffness and a complementary tunable hydrogel culture system to examine how matrix stiffness influences HCC cell proliferation and DNA repair. Our data show that matrix stiffness substantially augments DNA repair capacity and confers radiotherapeutic resistance in HCC cells. Moreover, we uncovered a non-traditional, non-enzymatic function of the glycolytic regulator PFKFB3 in transducing these mechanosensitive signals.

Materials and Methods

Cell culture and HCC tissue samples

MHCC97H cells were sourced from the Liver Cancer Institute of Zhongshan Hospital, Fudan University, Shanghai. Hep3B cells and McA-RH7777 cells were acquired from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology and Shanghai Zishi Biotech Co., Ltd., respectively. MHCC97H and McA-RH7777 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Biowest) and 1% penicillin/streptomycin (Gibco). Hep3B cells were cultured in Minimum Essential Medium (Gibco) containing 12.5% FBS and 1% penicillin/streptomycin. We collected clinical information and HCC tissue specimens from 87 patients who underwent curative liver resection at the Department of Liver Surgery, Zhongshan Hospital, Fudan University, in 2008. Follow-up continued until December 2022. **Table 1** summarizes the clinicopathologic characteristics of these patients and the associated statistical evaluations. The use of HCC tissues and corresponding clinical data was approved by the Ethics Committee of Zhongshan Hospital, Fudan University (B2024-359R).

Table 1. Relationship between PFKFB3 expression and clinicopathological features in tumor tissues of HCC patients.

Clinicopathological factors	PFKFB3 Low	PFKFB3 high	P-value
	(N = 44)	(N = 43)	
Gender			
Female	6 (13.6%)	9 (20.9%)	.537
Male	38 (86.4%)	34 (79.1%)	

Age (years)			
Mean (SD)	53.2 (12.7)	56.0 (12.6)	.294
HBV			
Negative	7 (15.9%)	10 (23.3%)	.553
Positive	37 (84.1%)	33 (76.7%)	
TB (umol/L)			
≤ 17.1	37 (84.1%)	36 (83.7%)	1.000
> 17.1	7 (15.9%)	7 (16.3%)	
ALT (U/L)			
Mean (SD)	37.7 (26.0)	39.7 (32.9)	.755
PT (s)			
Mean (SD)	13.1 (1.04)	12.7 (1.07)	.205
Albumin (g/L)			
Mean (SD)	3.96 (0.328)	3.87 (0.341)	
AFP (ng/mL)			
≤ 400	29 (65.9%)	27 (62.8%)	.936
> 400	15 (34.1%)	16 (37.2%)	
Tumor size (cm)			
Mean (SD)	5.57 (2.73)	7.53 (3.63)	0.006
Tumour number			
Mean (SD)	1.11 (0.387)	1.19 (0.450)	0.424
Thrombi			
No	30 (68.2%)	21 (48.8%)	.107
Yes	14 (31.8%)	22 (51.2%)	
TNM			
I-II	36 (81.8%)	30 (69.8%)	.288
III	8 (18.2%)	13 (30.2%)	
Histological grade			
I-II	34 (77.3%)	33 (76.7%)	1.000
III	10 (22.7%)	10 (23.3%)	
COL1 expression			
Mean (SD)	48.7 (18.0)	58.0 (11.6)	.005
LOX expression			
Mean (SD)	57.4 (18.3)	68.6 (9.48)	<.001
COL1&LOX expression			
COL1^{High}/LOX^{High}	14 (31.8%)	18 (41.9%)	.008
COL1^{High}/LOX^{Low}	3 (6.8%)	8 (18.6%)	
COL1^{Low}/LOX^{High}	3 (6.8%)	8 (18.6%)	
COL1^{Low}/LOX^{Low}	24 (54.5%)	9 (20.9%)	

Abbreviations: AFP = alpha-fetoprotein; ALT = alanine aminotransferase; COL1 = collagen 1; HBV = hepatitis B virus; HCC = hepatocellular carcinoma; LOX = lysyl oxidase; PFKFB3 = phosphofructokinase-2/fructose-2,6-bisphosphatase 3; PT = prothrombin time; TB = total bilirubin; TNM = tumour node metastasis.

Tunable stiffness hydrogel culture platform

Fibronectin-coated polyacrylamide gels were fabricated to create substrates with specific rigidity: 6 kPa (soft), 10 kPa (medium), and 16 kPa (stiff), prepared as outlined in an earlier report [28]. HCC cell suspensions were evenly distributed onto these gel surfaces and incubated at 37 °C for 2 to 3 hours to allow attachment. After complete

attachment, standard growth medium was gently introduced, and cells were thereafter maintained under routine culture conditions.

Generation of high-stiffness liver background HCC model in SD rats

All animal handling and procedures complied fully with the Institutional Animal Care and Use Committee regulations at Zhongshan Hospital, Fudan University. The high-liver-stiffness HCC rat model was produced according to the established protocol [28]. Briefly, young male SD rats (3–4 weeks of age, obtained from Shanghai Slack Laboratory Animal Company) first received abdominal subcutaneous injections of pure carbon tetrachloride (3 mL/kg) in week one. From week 2, they received twice-weekly injections (Mondays and Thursdays) of 50% carbon tetrachloride diluted in olive oil (2 mL/kg) for 12 weeks to establish elevated liver stiffness.

Subsequently, either X-ray-irradiated McA-RH7777 cells (6×10^6) or untreated McA-RH7777 cells (6×10^6) were suspended separately in 100 μ L 1 $\times$ PBS and combined with 100 μ L Matrigel (low growth factor type, Corning). The mixtures were injected directly under the liver capsule. Dexamethasone (6.5 mg/kg, intramuscular) was given on the day of surgery, plus one day before and one day after.

Models were stratified into four experimental arms according to liver stiffness and irradiation status: normal stiffness control (group N), high stiffness (group H), normal stiffness with irradiation (group N+IR), and high stiffness with irradiation (group H+IR). After 17 days, tumors were excised, measured for length and short diameter, weighed, and volumes computed as $0.5 \times \text{length} \times (\text{short diameter})^2$.

X-ray exposure procedure

Cells scheduled for irradiation were moved to the linear accelerator suite. Dishes were placed on a 1-cm soft rubber layer to promote even dose distribution. Irradiation was delivered at doses ranging from 2 to 14 Gy using a Siemens Oncor linear accelerator with 6 MV X-rays, a dose rate of 300 MU/min, and an 180° gantry position at ambient temperature. Following exposure, cells were promptly returned to the 37°C incubator.

shRNA lentiviral gene silencing

shRNA sequence selection, plasmid construction, and lentivirus production were outsourced to Shanghai Jikai Gene Co., Ltd. Target fragments for PIEZO1 and ITGB1 were cloned into GV112 vectors. In contrast, those for PFKFB3 and NEDD4 were cloned into GV115 vectors. About 1×10^5 HCC cells per well were plated in 6-well plates with 1 mL of complete medium. At 30–40% confluency, transduction was performed by adding

medium, 40 μ L of Hitrans GP reagent, and appropriate lentivirus. MOI was 5 for ITGB1 shRNA and 20 for the rest. Knockdown validation was done via immunoblotting. Target sequences used: PIEZO1 (ccCTGTGCATTGATTATCCCT), ITGB1 (CCTCCAGATGACATAGAAA), PFKFB3 (ctCCAATATCATGGAAGTTAA), NEDD4 (gcTGAACCTATACGGTTCAAAT).

Pharmacological treatments

Yoda1 (Piezo1 activator, MedChemExpress) was applied at 5 μ M for 48 h; GsMTx4 (Piezo1 blocker, Abcam) was applied at 2.5 μ M for 48 h. Proteasome inhibition was performed using MG132 (Cell Signaling Technology) at 10 μ M for 6 h to prevent PFKFB3 breakdown. Protein synthesis was blocked with cycloheximide (CHX, MedChemExpress) at 100 μ g/mL, with samples harvested at designated intervals. Autophagy inhibition was performed with chloroquine (CQ, MedChemExpress) at 10 μ M for 6 h.

Isolation of nuclear fractions

Nuclear protein was isolated using the Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher) according to the supplier's guidelines. Fresh cell pellets were washed in 1 $\times$ PBS, resuspended in ice-cold CER I, vortexed for 15 s, and kept on ice for 10 min. CER II was added, followed by 15 s vortexing, 2 min on ice, and centrifugation (16,000 g, 15 min). After supernatant removal and PBS wash, the pellet was resuspended in ice-cold NER, vortexed for 40 min, and centrifuged (16,000 g, 10 min) to collect nuclear protein.

Neutral comet assay for DNA damage

Harvested cells were resuspended in PBS (1×10^5 /mL). Melted LMAgarose (Trevigen), cooled to 37°C, was mixed with 50 μ L cell suspension and 500 μ L agarose. Fifty microliters of this blend were applied to CometSlides, solidified at 4°C for 10 min in a humid chamber, then lysed at 4°C for 40 min. Slides were equilibrated in neutral electrophoresis buffer (30 min, 4°C) and run at 21 V. After precipitation and fixation in 70% ethanol (30 min each, room temperature), slides were stained with DAPI/PI (Beyotime), imaged via fluorescence microscopy (Olympus), and quantified using Casplab_1.2.3b2.

Plasmid constructs and transfection

Expression plasmids for human PFKFB3-Vector, wild-type PFKFB3, PFKFB3-E3A/Q10A/S25A mutant, and PFKFB3-T339A/E343A/Q363A mutant were acquired from Shanghai GeneChem Co., Ltd. using the GV657 backbone. Transient transfection of HCC cells was performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

Protein-protein docking simulation

Docking experiments utilized Schrödinger Suite software. PDB structures for PFKFB3 (entry 2AXN) and XRCC6 (entry 1JEQ) were obtained from the RCSB Protein Data Bank. Structures were prepared using the Protein Preparation Wizard, ligands were prepared using LigPrep, and docking was performed in Prime mode. Binding affinities were evaluated with MM-GBSA calculations in the Prime module.

miRNA transfection

hsa-miR-199a-3p mimics, negative control mimics, inhibitors, and negative control inhibitors were sourced from RiboBio. Transfections were performed according to the Lipofectamine RNAiMAX (Invitrogen) protocol. Sequences:

mimic	NC
(UUUGUACUACACAAAAGUACUG),	hsa-miR-199a-3p
(ACAGUAGUCUGCACAUUGGUUA),	mimic
(CAGUACUUUUGUGUAGUACAAA),	inhibitor NC
(UAACCAAUGUGCAGACUACUGU).	hsa-miR-199a-3p inhibitor

Metabolic flux analysis (Seahorse)

Cells were plated at 4×10^4 cells/well. The next day, assay medium with 10 mM glucose was added. ECAR was recorded on a Seahorse XF96 analyzer (Agilent). Measurements included basal levels followed by injections of oligomycin (4 μ M) and 2-deoxyglucose (500 mM).

Data analysis and statistics

Analyses were conducted in GraphPad Prism version 8.0. Results are reported as mean $\pm$ standard deviation. Two-group comparisons of continuous data used a two-tailed unpaired Student's t-test. Multigroup data involving two variables were analyzed using a two-way ANOVA.

Categorical variables were evaluated with a Pearson chi-square test. Statistical significance was defined as $P < 0.05$.

Results and Discussion

Elevated matrix stiffness drives HCC tumor expansion and confers resistance to radiotherapy

To assess the effects of mechanical stiffness on tumor behavior in irradiated versus non-irradiated HCC cells, we used SD rat models with controlled liver rigidity induced by combined methods [28]. As depicted in the study schematic, chronic CCl₄ administration resulted in markedly stiffened livers, a feature previously confirmed in our earlier work [28]. Control rats with physiological stiffness and high-stiffness rats then underwent intrahepatic implantation of Matrigel-embedded McA-RH7777 cells that were either pre-exposed to X-rays or left untreated, along with perioperative dexamethasone support.

We selected a 4 Gy dose, achieving roughly 80% viability. Seventeen days after implantation, tumors were retrieved from the four cohorts: normal stiffness (N), high stiffness (H), normal stiffness plus irradiation (N+IR), and high stiffness plus irradiation (H+IR). Tumors in the high-stiffness setting (group H) displayed substantially larger weights and volumes than those in normal-stiffness livers (group N) (**Figure 1a**), confirming accelerated progression under rigid conditions in line with previous reports [29]. Irradiation markedly reduced tumor burden in both backgrounds, yet the residual tumors arising from irradiated cells grew more robustly in high-stiffness livers than in normal ones, as evidenced by greater fold changes in size and mass (**Figure 1a**).

Ki67 immunostaining revealed stronger proliferative activity in high-stiffness tumors (H vs N; H+IR vs N+IR) (**Figure 1b**), while TUNEL staining indicated correspondingly lower apoptosis rates. Taken together, these data establish that heightened liver matrix stiffness not only accelerates HCC tumor development but also bolsters the ability of cancer cells to withstand radiation-induced DNA damage, thereby weakening overall radiotherapy effectiveness.

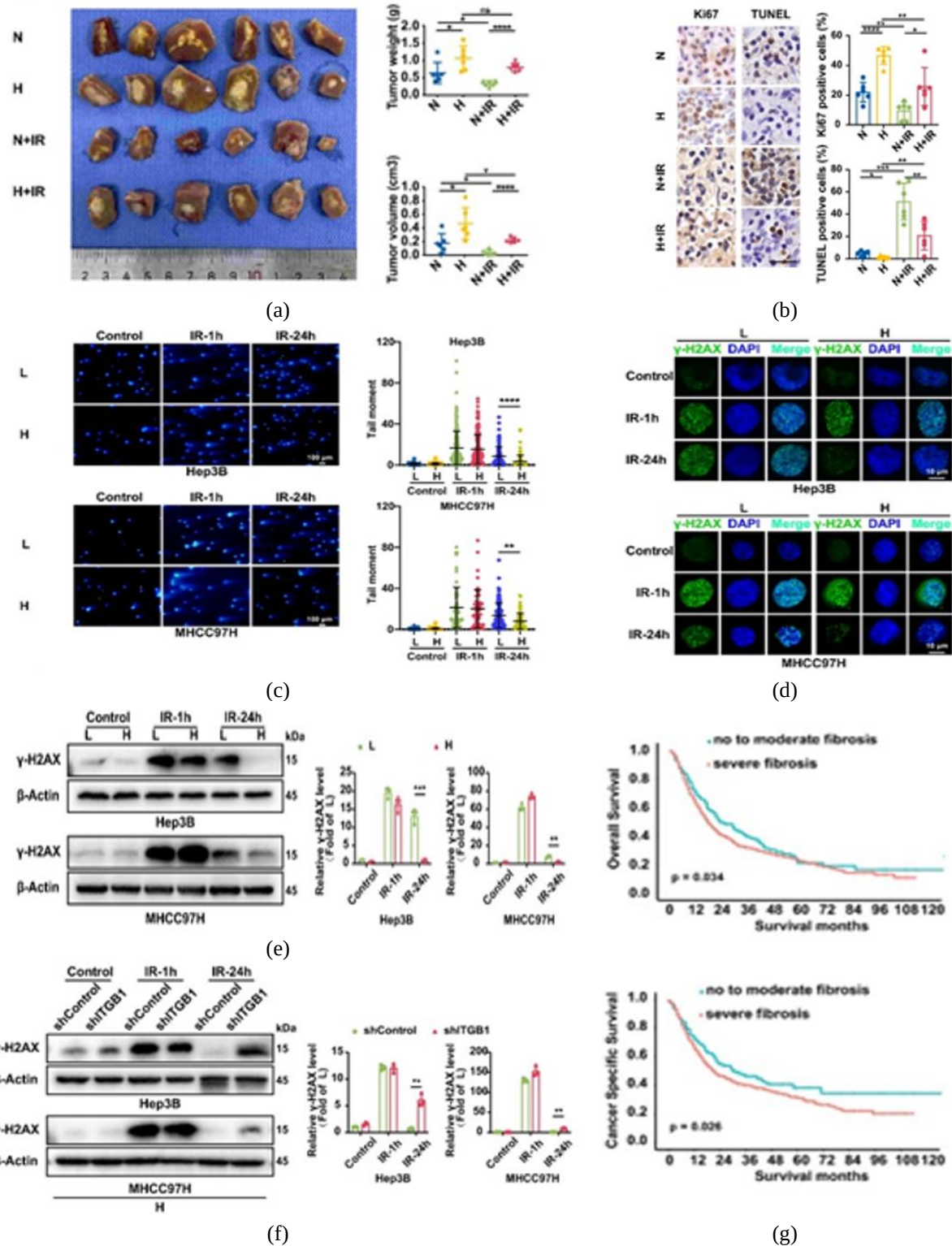


Figure 1. Matrix rigidity promotes HCC tumor expansion and reduces radiotherapy sensitivity

(a) HCC models in SD rats with normal or elevated liver stiffness were created to examine how mechanical cues influence tumor development from irradiated versus non-

irradiated McA-RH7777 cells. N: normal stiffness group; H: high stiffness group; N+IR: normal stiffness plus irradiation; H+IR: high stiffness plus irradiation. Tumor

weight and volume were compared across groups; (b) Left: Representative immunohistochemistry staining for Ki67 and TUNEL. Right: Quantification of Ki67-positive and TUNEL-positive cells in tumor sections from each group. Scale bar: 20 μ m; (c) Neutral comet assay showing tail moments in HCC cells cultured on soft (6 kPa) and stiff (16 kPa) substrates at 1 h and 24 h post-irradiation (scale bar: 100 μ m), with quantification of tail moment per cell ($n > 50$ cells); (d) Immunofluorescence images of γ -H2AX foci in HCC cells on low- and high-stiffness substrates at 1 h and 24 h after irradiation. Nuclei counterstained with DAPI (blue). Scale bar: 10 μ m; (e) Western blot analysis of γ -H2AX levels in HCC cells on low- and high-stiffness substrates at the indicated time points post-irradiation. L: low-stiffness (6 kPa); H: high-stiffness (16 kPa); (f) γ -H2AX expression in integrin β 1-knockdown HCC cells cultured on high-stiffness substrates at 1 h and 24 h after irradiation; (g) Kaplan-Meier curves for overall survival and cancer-specific survival in HCC patients undergoing radiotherapy, stratified by fibrosis stage from the SEER database. IR: irradiation. shControl: control vector; shITGB1: integrin β 1 knockdown. Data shown as mean (SD); * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$, ns: not significant; two-tailed Student's t-test.

To confirm the *in vivo* observations that rigid matrix conditions confer radioprotection, we established *in vitro* culture systems using polyacrylamide gels of 6 kPa (soft, mimicking normal liver) and 16 kPa (stiff, mimicking cirrhotic liver) as reported earlier [29, 30]. We chose an X-ray dose of 4 Gy based on the dose-response curve, which yielded approximately 75% cell survival. Rigid substrates significantly enhanced post-irradiation survival of HCC cells, highlighting the direct contribution of biomechanical forces to treatment resistance. In contrast, silencing integrin β 1, a key mechanosensor, substantially reduced survival of irradiated cells specifically on stiff gels. These findings demonstrate that matrix rigidity directly improves survival following radiation exposure.

Neutral comet assays further revealed faster resolution of DNA lesions in cells on stiff substrates. While both conditions showed prominent tail moments at 1 h post-irradiation, cells on high-stiffness gels exhibited a markedly sharper decline in tail moment by 24 h, signifying more efficient DNA repair (**Figure 1c**), consistent with animal model results. Complementary

assessment of γ -H2AX, a marker of double-strand breaks, showed strong induction of foci and protein levels at 1 h in both stiffness groups. However, by 24 h, both γ -H2AX foci counts and overall expression were substantially lower on stiff substrates compared to soft ones (**Figures 1d and 1e**). These patterns aligned with comet assay data, reinforcing that elevated stiffness accelerates DNA damage repair in HCC cells. This protective effect depended on integrin β 1, as its depletion significantly hindered repair on stiff substrates (**Figure 1f**). Overall, matrix stiffness acts as a potent extrinsic factor that bolsters cell survival after genotoxic insult and contributes to radiotherapy resistance in HCC.

We additionally analyzed clinical records from the SEER database to assess fibrosis stage as a surrogate for stiffness in relation to radiotherapy outcomes. Using the Ishak scoring system [31], we categorized patients receiving radiotherapy as Ishak 0–4 (none to moderate fibrosis) or Ishak 5–6 (advanced to severe fibrosis). Overall survival was significantly longer in the Ishak 0–4 group than in the Ishak 5–6 group (median 23 vs. 17 months; $P = 0.034$) (**Figure 1g**). Cancer-specific survival showed a similar advantage (median 31 vs. 21 months; $P = 0.026$) (**Figure 1g**). These patient data support the notion that greater matrix stiffness diminishes radiotherapy efficacy, aligning with experimental findings from animal and cell-based models. Univariate and multivariate analyses identified independent prognostic factors for overall survival (OS) and cancer-specific survival (CSS). In multivariate models, Ishak score 0–4 was associated with reduced mortality risk compared to score 5–6 for OS (HR = 0.73, 95% CI: 0.60–0.90, $P = 0.003$) and for CSS (HR = 0.70, 95% CI: 0.56–0.88, $P = 0.002$). In parallel, HCC cells cultured on progressively stiffer substrates showed enhanced proliferation, increased viability, and higher Ki-67 levels (**Figures 2a–2c**). Expression of proliferation-related proteins—including PCNA, cyclin D1 (CCND1), cyclin D3 (CCND3), CDK2, and CDK4—also rose with increasing matrix stiffness (**Figure 2d**). These *in vitro* results corroborate the tumor-promoting effects seen in animal studies. Conversely, interfering with mechanosensors such as integrin β 1 or Piezo1 on stiff substrates led to marked reductions in Ki67 (**Figure 2e**), PCNA, and CCND1 (**Figure 2f**), further confirming that mechanical stiffness drives proliferative capacity in HCC cells.

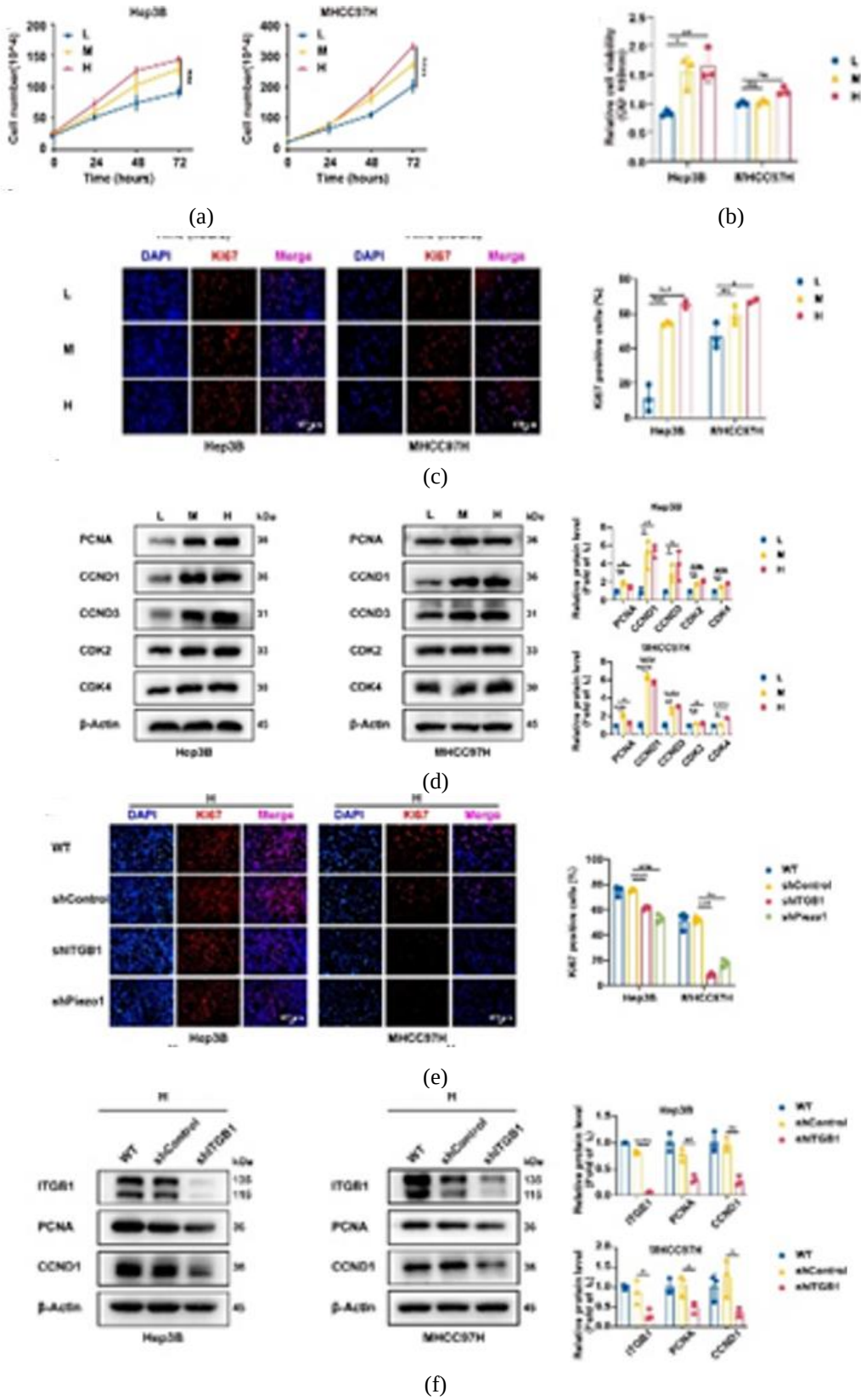


Figure 2. Elevated matrix stiffness stimulates proliferation of HCC cells

(a) Growth curves of HCC cells cultured on substrates of varying rigidity (L: 6 kPa; M: 10 kPa; H: 16 kPa) over time; (b) Cell viability assessed by CCK-8 assay after 72 hours on substrates with different stiffness levels (L: 6 kPa; M: 10 kPa; H: 16 kPa); (c) Representative immunofluorescence staining for Ki67 in HCC cells grown on low-, medium-, and high-stiffness substrates, with quantification of Ki67-positive cells (DAPI counterstain for nuclei). Scale bar: 100 μ m; (d) Protein levels of proliferation markers in HCC cells on substrates of increasing stiffness. PCNA: proliferating cell nuclear antigen; CCND1: cyclin D1; CCND3: cyclin D3; CDK2: cyclin-dependent kinase 2; CDK4: cyclin-dependent kinase 4; (e) Immunofluorescence images and quantification of Ki67 in HCC cells expressing shITGB1 or shPiezo1 on high-stiffness substrates (DAPI nuclear stain). Scale bar: 100 μ m; (f) Integrin β 1 knockdown reduces PCNA and CCND1 protein expression in cells on rigid substrates. L: low-stiffness (6 kPa); M: medium-stiffness (10 kPa); H: high-stiffness (16 kPa). WT: wild-type; shControl: control vector; shITGB1: integrin β 1 knockdown; shPiezo1: Piezo1 knockdown. Data presented as mean (SD); * P < .05, ** P < .01, *** P < .001, **** P < .0001, ns: not significant. Statistical tests: two-way ANOVA for (A), two-tailed Student's t-test for others.

PFKFB3 mediates stiffness-induced proliferation and radiotherapy resistance in HCC

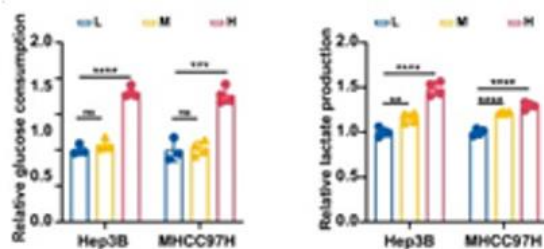
Given the established contributions of glycolytic enzymes to tumor cell growth and survival [32], along with their emerging non-enzymatic roles [27], we hypothesized that these enzymes could link matrix stiffness to enhanced proliferation and treatment resistance. Measurements showed markedly higher glucose uptake and lactate output in cells cultured on rigid substrates (**Figure 3a**). Silencing either integrin β 1 or Piezo1 effectively blocked the stiffness-driven increases in glucose consumption and lactate production,

confirming that biomechanical signals elevate glycolytic activity to fuel tumor expansion.

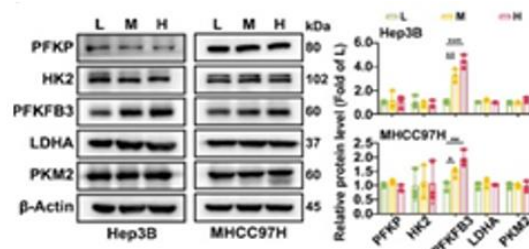
Since glycolytic rate is commonly governed by the abundance of rate-controlling enzymes [25, 26], we examined whether stiffness alters their expression. Among several key glycolytic proteins tested, only PFKFB3 displayed robust and selective induction under stiff conditions, whereas PFKP, HK2, LDHA, and PKM2 levels remained unchanged (**Figure 3b**). PFKFB3 depletion clearly reduced the extracellular acidification rate (ECAR), a readout of glycolytic flux (**Figure 3c**), indicating that PFKFB3 is the primary mediator of stiffness-enhanced glycolysis in HCC cells.

This upregulation of PFKFB3 on rigid substrates required intact mechanosensing through integrin β 1 and Piezo1. Knockdown of either sensor prevented the stiffness-dependent rise in PFKFB3 (**Figure 3d**). Pharmacologically, treatment with the Piezo1 agonist Yoda1 on soft substrates was sufficient to increase PFKFB3 levels, while the antagonist GsMTx4 on stiff substrates abolished its induction (**Figures 3e and 3f**). These results establish that matrix stiffness elevates PFKFB3 expression via integrin β 1 and Piezo1 signaling pathways.

Functional studies further demonstrated that PFKFB3 is necessary for the proliferative response to stiffness. PFKFB3 knockdown on high-stiffness substrates significantly reduced Ki67 levels (**Figure 3g**) and decreased expression of proliferation regulators, including PCNA and CCND1 (**Figure 3h**). Clinically, PFKFB3 protein was notably elevated in human HCC samples classified as high-stiffness (based on COL1^{High}/LOX^{High}, $n=32$) compared to low-stiffness tumors (COL1^{Low}/LOX^{Low}, $n=33$) (**Figure 3i**). Higher PFKFB3 expression also correlated with larger tumor size in clinicopathologic analysis (**Table 1**). Collectively, these data identify PFKFB3 — an important regulator of glycolysis [33] — as a central driver of matrix stiffness-promoted HCC cell proliferation and tumor growth.



(a)



(b)

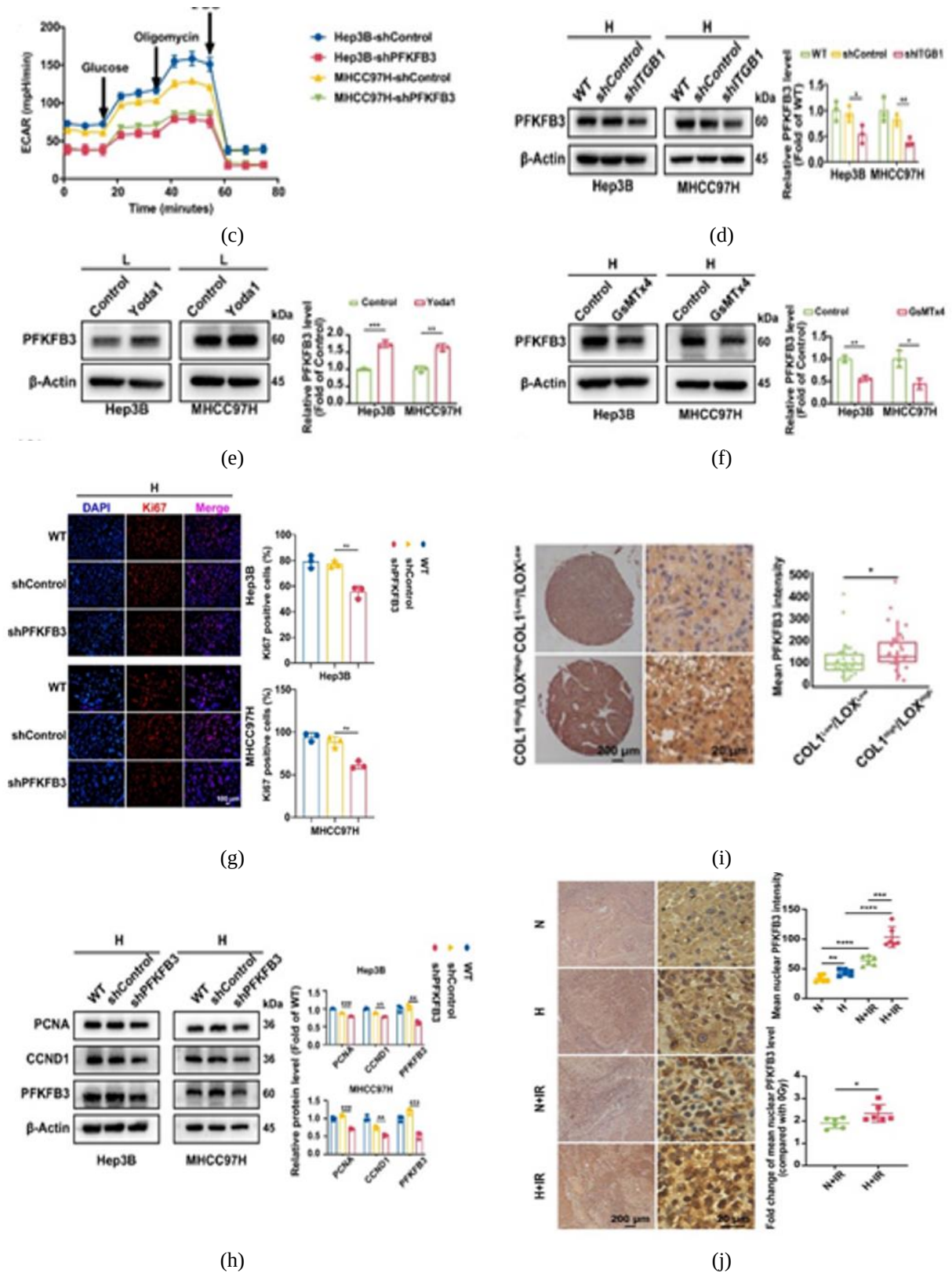
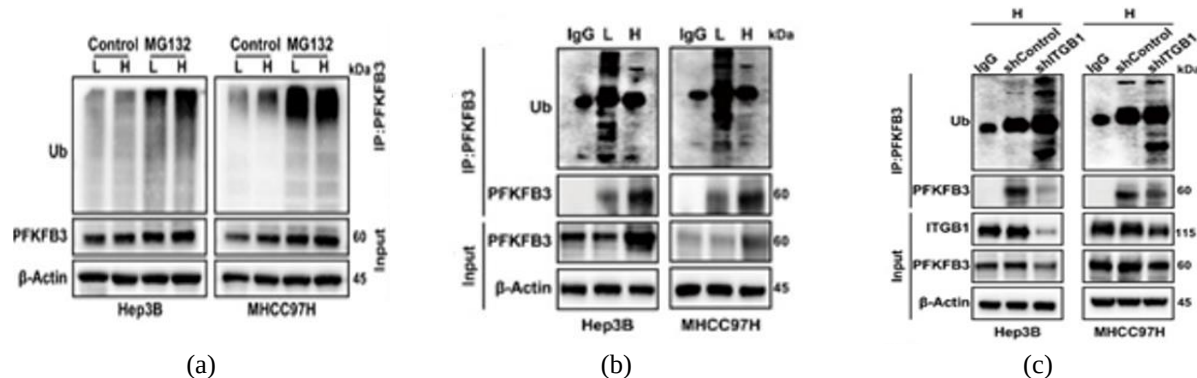


Figure 3. The metabolic regulator PFKFB3 contributes to matrix stiffness-driven proliferation and radiotherapy resistance in HCC

(a) Glucose consumption and lactate production in HCC cells cultured on substrates of varying stiffness (L: 6 kPa; M: 10 kPa; H: 16 kPa), normalized to cell counts; (b) Protein expression of major glycolytic enzymes in HCC cells on low-, medium-, and high-stiffness substrates. PFKP: phosphofructokinase platelet-type; HK2: hexokinase 2; PFKFB3: phosphofructokinase-2/fructose-2,6-bisphosphatase 3; LDHA: lactate dehydrogenase A; PKM2: pyruvate kinase M2; (c) Glycolytic flux measured by extracellular acidification rate (ECAR) in PFKFB3-knockdown cells; (d) PFKFB3 levels in integrin β 1-silenced HCC cells on high-stiffness substrates; (e) PFKFB3 expression in cells on soft substrates following 48-hour treatment with Yoda1 (5 μ M); (f) PFKFB3 expression in cells on stiff substrates after 48-hour GsMTx4 treatment (2.5 μ M); (g) Immunofluorescence staining and quantification of Ki67 in PFKFB3-knockdown HCC cells on high-stiffness substrates (DAPI nuclear counterstain). Scale bar: 100 μ m; (h) Levels of proliferation-related proteins (PCNA and CCND1) in shPFKFB3 cells under high-stiffness conditions; (i) PFKFB3 expression in clinical HCC specimens from high-stiffness (COL1^{High}/LOX^{High}, n=32) versus low-stiffness (COL1^{Low}/LOX^{Low}, n=33) groups. Scale bar: 200 μ m (left), 20 μ m (right); (j) Immunohistochemical analysis of PFKFB3 expression and localization in rat HCC tissues from groups N, H, N+IR, and H+IR. Scale bar: 200 μ m (left), 20 μ m (right). Upper right: quantification of mean nuclear PFKFB3 intensity. Lower right: fold change in nuclear PFKFB3 intensity in irradiated tumors relative to non-irradiated controls under normal or high stiffness backgrounds. N:

normal stiffness; H: high stiffness; N+IR: normal stiffness + irradiation; H+IR: high stiffness + irradiation. L: 6 kPa; M: 10 kPa; H: 16 kPa. shControl: control vector; WT: wild-type; shITGB1: integrin β 1 knockdown; shPFKFB3: PFKFB3 knockdown. Data shown as mean (SD); *P < .05, **P < .01, ***P < .001, ****P < .0001, ns: not significant; two-tailed Student's t-test.

Beyond their conventional roles in carbohydrate metabolism, certain metabolic enzymes can influence cellular behaviors and pathological processes through nuclear shuttling independent of their catalytic activity [27, 34]. We therefore investigated whether PFKFB3 could translocate to the nucleus to mediate stiffness-dependent radiotherapy resistance in HCC. Analysis of PFKFB3 localization in tumor tissues from the four animal model groups revealed patterns consistent with in vitro observations. Overall PFKFB3 abundance was substantially higher in tumors from high-stiffness livers compared to normal-stiffness livers (**Figure 4g**), (note: figure reference as in original). In normal-stiffness tumors, PFKFB3 was predominantly cytoplasmic, whereas in high-stiffness tumors it was present in both cytoplasm and nuclei (**Figure 3j**). Critically, nuclear PFKFB3 accumulation was most pronounced in the H+IR group, exceeding levels seen in H and N+IR groups. This demonstrates enhanced nuclear translocation of PFKFB3 specifically in irradiated HCC cells under high-matrix-stiffness conditions (**Figure 3j**).



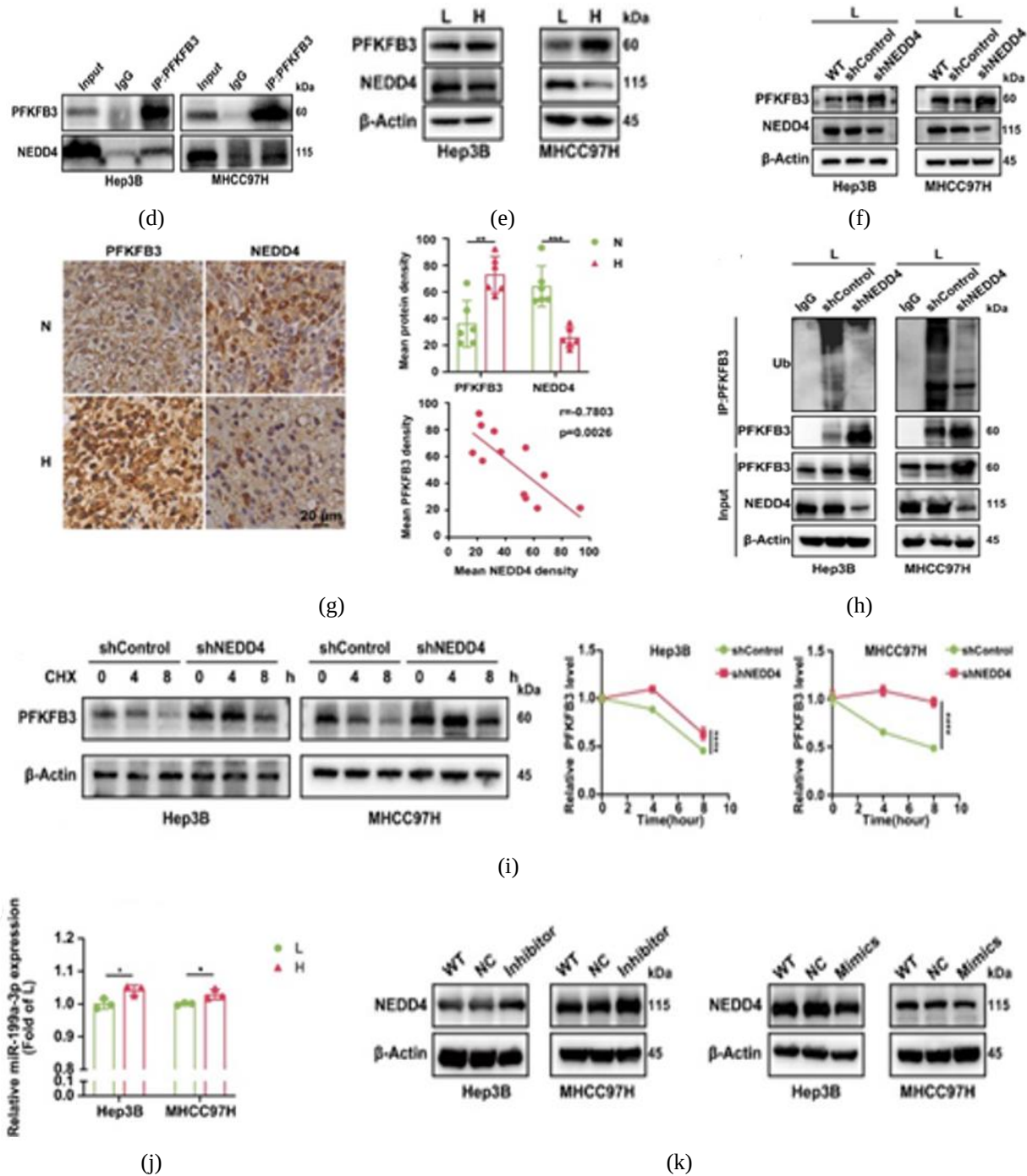


Figure 4. Rigid matrix conditions elevate PFKFB3 protein levels in HCC cells by reducing its ubiquitination

(a) PFKFB3 and ubiquitin levels in HCC cells on soft and stiff substrates following 6-hour MG132 treatment (10 μ M). Control: DMSO vehicle. L: 6 kPa; H: 16 kPa; (b) Polyubiquitinated PFKFB3 detected after immunoprecipitation of PFKFB3 from whole-cell lysates of cells cultured on low- and high-stiffness substrates (L: 6 kPa; H: 16 kPa); (c) Polyubiquitinated PFKFB3 levels

following PFKFB3 pull-down in integrin β 1-knockdown cells on stiff substrates (H: 16 kPa); (d) Co-immunoprecipitation and immunoblotting confirming physical interaction between NEDD4 and PFKFB3; (e) Protein expression of PFKFB3 and NEDD4 (neural precursor cell expressed, developmentally down-regulated 4) in HCC cells on substrates of different

stiffness (L: 6 kPa; H: 16 kPa); (f) PFKFB3 abundance in NEDD4-knockdown HCC cells on soft substrates (L: 6 kPa); (g) Representative IHC staining of PFKFB3 and NEDD4 in rat HCC tissues from normal and high liver stiffness groups (left). Scale bar: 20 μ m. Quantification of mean staining intensity (upper right). Pearson correlation analysis between PFKFB3 and NEDD4 expression (lower right). N: normal stiffness group; H: high stiffness group; (h) Polyubiquitinated PFKFB3 after pull-down in NEDD4-knockdown cells on low-stiffness substrates; (i) PFKFB3 protein stability in shNEDD4 cells treated with CHX (100 μ g/mL) and harvested at indicated times; (j) Relative miR-199a-3p levels in HCC cells on substrates of varying stiffness. (k) PFKFB3 expression in cells transfected with miR-199a-3p inhibitor or mimics. Ub: ubiquitin; IgG: isotype control; shControl: empty vector; shITGB1: integrin β 1 knockdown; shNEDD4: NEDD4 knockdown. Data presented as mean (SD); statistical tests as indicated; * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$.

These observations suggest that stiff matrices partially enhance radiation resistance by promoting nuclear translocation of PFKFB3 in HCC cells.

Rigid matrix conditions elevate PFKFB3 protein abundance in HCC cells through suppression of ubiquitination

Although high stiffness strongly increased PFKFB3 protein levels (**Figure 3b**), it did not affect PFKFB3 mRNA expression. This discrepancy pointed to post-translational regulation, likely involving ubiquitination. Proteasome blockade with MG132 elevated both PFKFB3 protein and total ubiquitin content regardless of substrate stiffness (**Figure 4a**). Immunoprecipitation experiments showed a marked reduction in PFKFB3 polyubiquitination in cells grown on stiff substrates (**Figure 4b**). CHX chase experiments further showed delayed PFKFB3 turnover under high stiffness, confirming that rigid conditions inhibit ubiquitination and thereby stabilize the protein. Treatment with chloroquine (CQ) failed to restore PFKFB3 levels on soft substrates, ruling out a significant role for lysosomal degradation. In contrast, knockdown of integrin β 1 or Piezo1 restored high ubiquitination and reduced PFKFB3 protein on stiff substrates (**Figure 4c**). Thus, matrix stiffness upregulates PFKFB3 primarily by blocking ubiquitin-proteasome-mediated degradation.

E3 ubiquitin ligases facilitate attachment of ubiquitin chains to target proteins, marking them for proteasomal

destruction [35]. To identify the responsible ligase for PFKFB3, we performed immunoprecipitation followed by LC-MS/MS to detect interacting partners in HCC cells. From 2126 identified proteins, 15 overlapped with known human E3 ligases curated in the NHLBI database (<https://esbl.nhlbi.nih.gov/Databases/KSBP2/Targets/Lists/E3-ligases/>). Applying a PSM threshold >4 narrowed candidates to four: PPIL2, PRPF19, TRIM21, and NEDD4. We excluded PPIL2 and TRIM21 due to low specificity (P3/IgG ratio ≤ 1). Survival analysis using the Kaplan-Meier Plotter for liver hepatocellular carcinoma (<http://kmplot.com/>) revealed that only elevated NEDD4 expression was associated with better prognosis. Co-immunoprecipitation confirmed endogenous binding between PFKFB3 and NEDD4 (**Figure 4d**).

NEDD4 levels were significantly lower on stiff substrates, inversely correlating with PFKFB3 upregulation (**Figure 4e**). NEDD4 knockdown on soft substrates increased PFKFB3 protein without altering mRNA (**Figure 4f**), supporting its role as the specific E3 ligase targeted by stiffness signals. In the SD rat HCC model, IHC analysis showed a strong negative correlation between NEDD4 and PFKFB3, with high-stiffness tumors displaying high PFKFB3 and low NEDD4 (**Figure 4g**). Functionally, NEDD4 depletion reduced PFKFB3 polyubiquitination and prolonged its half-life (**Figures 4h and 4i**).

Downregulation of integrin β 1 or Piezo1 on stiff substrates also restored NEDD4 expression. We next explored upstream control of NEDD4. Previous studies indicated that miR-199a-3p suppresses NEDD4 [36] and responds positively to mechanical cues [37]. Indeed, miR-199a-3p levels rose on stiff substrates (**Figure 4j**) and fell upon integrin β 1 or Piezo1 knockdown. Inhibiting miR-199a-3p increased NEDD4 protein, while mimicking it decreased NEDD4 (**Figure 4k**). Therefore, elevated matrix stiffness downregulates NEDD4 by inducing miR-199a-3p, ultimately stabilizing PFKFB3.

Nuclear shuttling of PFKFB3 participates in stiffness-enhanced DNA repair in HCC

Observations of PFKFB3 localization in animal model tumors (**Figure 3j**) suggested its nuclear translocation might contribute to stiffness-mediated radioprotection. We examined nuclear fractions from irradiated HCC cells cultured on stiff versus soft substrates. PFKFB3 was detectable in nuclear extracts under both conditions, but nuclear accumulation was substantially greater on high-stiffness substrates (**Figure 5a**). Immunofluorescence

imaging yielded consistent results, with a more prominent nuclear PFKFB3 signal in irradiated cells on rigid gels (**Figure 5b**). These data corroborate the

enhanced nuclear translocation observed in vivo and indicate that high matrix stiffness strongly promotes PFKFB3 nuclear entry in irradiated HCC cells.

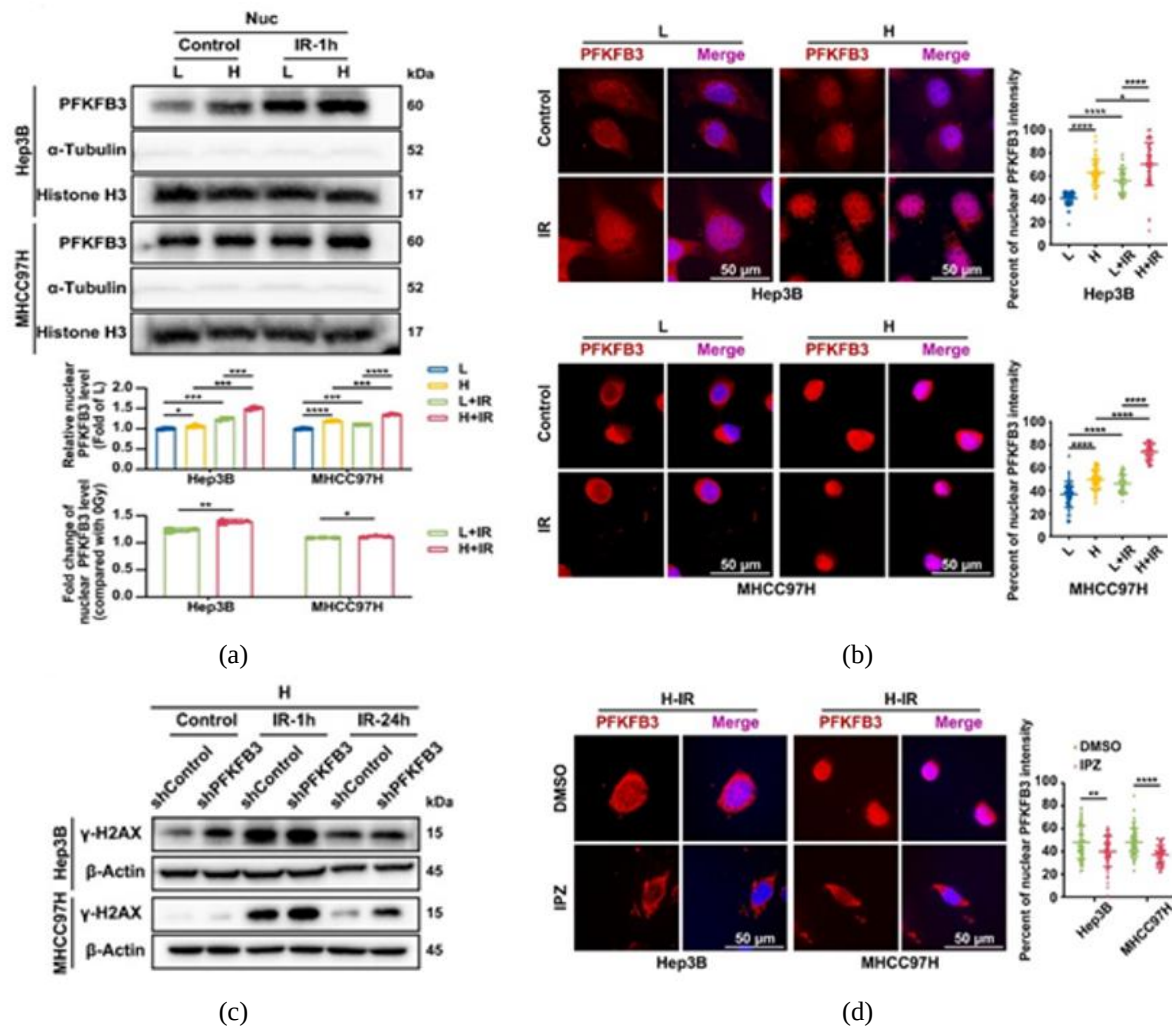


Figure 5. Nuclear shuttling of PFKFB3 supports matrix stiffness-enhanced DNA repair in HCC cells

(a) Top: Nuclear PFKFB3 levels in irradiated HCC cells (1 h post-irradiation) cultured on soft and stiff substrates. Nuc: nuclear fraction. Middle: Quantification of nuclear PFKFB3 normalized to Histone H3. Bottom: Fold change in nuclear PFKFB3 in irradiated cells relative to non-irradiated controls on each substrate; (b) Representative immunofluorescence images of PFKFB3 localization in irradiated HCC cells on low- and high-stiffness substrates (1 h post-irradiation), with quantification of nuclear PFKFB3 intensity per cell ($n \geq 50$ cells). Scale bar: 50 μm ; (c) γ -H2AX protein levels in PFKFB3-knockdown HCC cells on high-stiffness substrates at 1 h and 24 h after irradiation; (d) Effect of importazole (IPZ,

20 μM) on PFKFB3 nuclear translocation in irradiated cells on stiff substrates. Scale bar: 50 μm . L: 6 kPa; H: 16 kPa. Values shown as mean (SD); * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$; two-tailed Student's *t*-test. Further experiments confirmed the link between PFKFB3 nuclear translocation and DNA repair efficiency. PFKFB3 knockdown reduced survival of irradiated HCC cells on stiff substrates and impaired DNA damage resolution under these conditions (**Figure 5c**). These findings establish PFKFB3 as a key mediator of stiffness-induced DNA repair. Since PFKFB3 nuclear import primarily occurs via the importin α/β pathway [38], we used importazole, a selective importin β

inhibitor, on irradiated cells cultured on high-stiffness substrates. Both immunofluorescence and nuclear fractionation showed that importazole partially blocked PFKFB3 nuclear entry (**Figure 5d**). Consequently, importazole treatment also diminished DNA repair capacity on stiff substrates.

The PFKFB3 enzymatic inhibitor 3PO reduced glucose consumption and lactate production on stiff substrates but did not impair DNA repair ability.

In summary, nuclear translocation of PFKFB3 plays an essential role in matrix stiffness-regulated DNA damage repair.

Nuclear PFKFB3 interacts with Ku70 to facilitate DNA damage repair

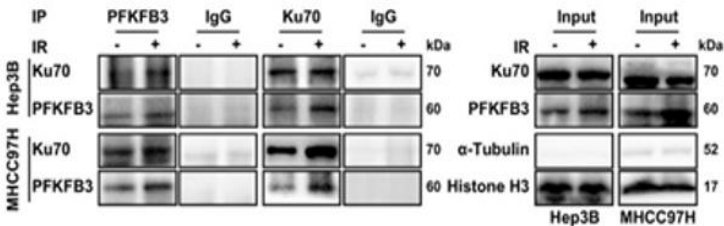
To elucidate how nuclear PFKFB3 contributes to DNA repair, we performed immunoprecipitation coupled with mass spectrometry on nuclear extracts from irradiated HCC cells to identify potential interactors. Using a PSM

> 4 cutoff, we identified 1005 nuclear proteins associated with PFKFB3. Cross-referencing with KEGG pathway gene sets for homologous recombination (HR) and non-homologous end joining (NHEJ) yielded four overlapping candidates: PRKDC, XRCC5, SSB, and XRCC6 (**Figure 6a**). Comparison with IgG control pull-downs indicated that PRKDC, XRCC5, and SSB were likely non-specific (P3/IgG ratios of 0.98, 0.88, and 1.33, respectively). XRCC6 (also known as Ku70) showed negligible signal in the IgG control, making it the strongest candidate for specific interaction with PFKFB3.

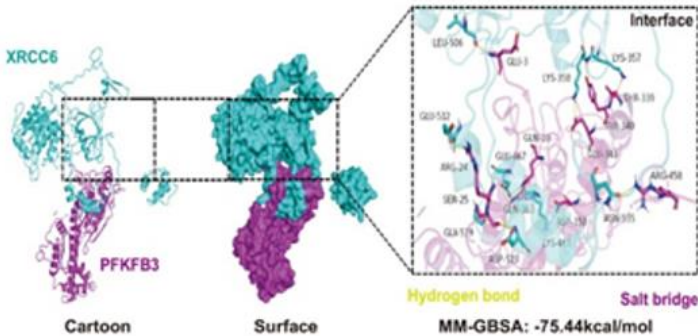
Reciprocal co-immunoprecipitation experiments in nuclear fractions of irradiated HCC cells confirmed endogenous complex formation between PFKFB3 and Ku70, along with XRCC4 (**Figure 6b**). This suggests that PFKFB3 is involved in the NHEJ repair pathway. Notably, the interaction between PFKFB3 and Ku70 was strengthened following X-ray irradiation (**Figure 6b**).

Protein name	#Peptide IgG	#Peptide P3	Peptide P3/IgG
PRKDC	65	64	0.98
XRCC6		15	
XRCC5	8	7	0.88
SSB	3	4	1.33

(a)



(b)



(c)

Hydrogen bond		
PFKFB3 (ligand)	Mutant	MM-GBSA (kcal/mol)
Wild-type	-	-75.44
B:Arg 458	Ala458	-74.62
B:Ser 25	Ala25	-73.93
B:Arg 24	Ala24	-74.83
B:Glu3	Ala3	-72.20
B:Gln 10	Ala10	-72.16
R:Gln 363	Ala363	-67.98
B:Tyr 340	Ala340	-66.07
B:Glu 343	Ala343	-64.91
B:Thr 339	Ala339	-60.29
Salt bridge		
PFKFB3 (ligand)	Mutant	MM-GBSA (kcal/mol)
B:Asp 350	Ala350	-69.18

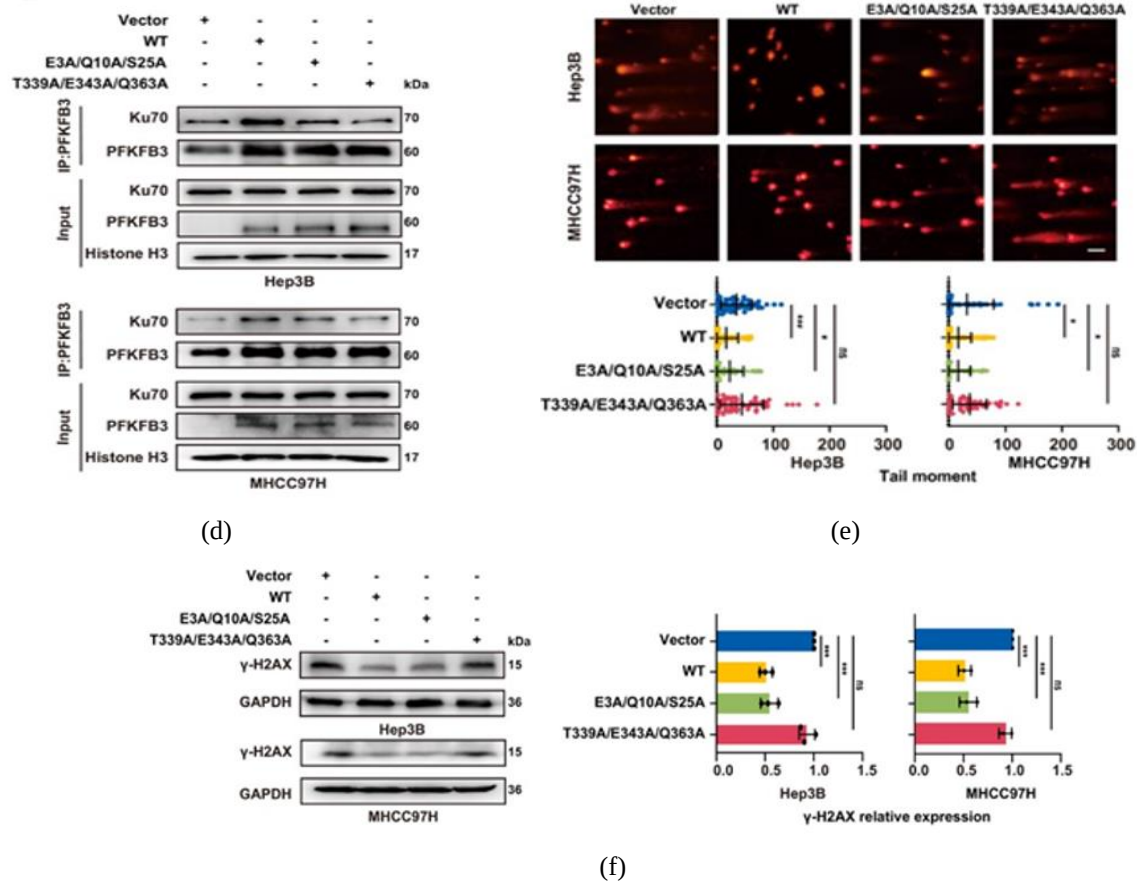


Figure 6. PFKFB3 partners with Ku70 during DNA damage repair

(a) Potential DNA repair factors associated with PFKFB3 in irradiated HCC cells, detected through immunoprecipitation-mass spectrometry. IgG: control antibody; P3: PFKFB3-specific antibody; (b) Western blot results from anti-PFKFB3 or anti-Ku70 pull-downs using nuclear lysates prepared 2 hours after irradiation; (c) Left panel: Computational docking model illustrating the binding interface between PFKFB3 (purple, PDB code 2AXN) and Ku70/XRCC6 (cyan, PDB code 1JEQ) via the Schrödinger platform. Right panel: Impact on MM-GBSA binding energies following alanine substitution at predicted key interaction residues (hydrogen bonds and salt bridges); (d) Co-immunoprecipitation experiments testing Ku70 association with different PFKFB3 variants (empty vector, wild-type, E3A/Q10A/S25A mutant, and T339A/E343A/Q363A mutant) in HCC cells; (e) Neutral comet assay results and tail moment quantification ($n > 50$ cells per condition) evaluating residual DNA damage 24 hours post-irradiation in cells expressing the various PFKFB3 constructs. Scale bar: 100 μ m; (f) Western blot

detection of γ -H2AX in HCC cells transfected with the indicated PFKFB3 plasmids 24 hours after irradiation. Data expressed as mean (SD); * $P < .05$, *** $P < .001$, **** $P < .0001$, ns: not significant; two-tailed Student's t-test.

To establish the functional importance of the PFKFB3–Ku70 physical association in repair processes, we conducted in-depth in silico docking analyses using Schrödinger software. The modeling predicted stable direct binding between the two proteins (**Figure 6c**). Alanine substitutions at critical contact points (Gln363, Tyr340, Glu343, Thr339) caused the largest decreases in calculated binding free energy compared with mutations at other positions (Arg458, Ser25, Arg24, Glu3, Gln10). We therefore engineered two PFKFB3 mutant versions: one disrupting the key interface (T339A/E343A/Q363A) and another at non-critical sites (E3A/Q10A/S25A). The T339A/E343A/Q363A variant exhibited greatly diminished binding to Ku70 in nuclear extracts (**Figure 6d**). Functional rescue experiments showed that both wild-type PFKFB3 and the E3A/Q10A/S25A mutant

effectively lowered residual DNA breaks, as indicated by reduced comet tail moments (**Figure 6e**) and decreased γ -H2AX (**Figure 6f**) at 24 hours after irradiation. However, the T339A/E343A/Q363A mutant provided no such protection, maintaining high tail moments and γ -H2AX levels comparable to the vector control. These data demonstrate that specific residues enabling PFKFB3 interaction with Ku70 are indispensable for its ability to mitigate irradiation-induced genomic damage.

Clinical importance of PFKFB3 expression in individuals with HCC

To extend the laboratory and animal observations to human disease, we evaluated PFKFB3 abundance in surgical HCC samples and examined its relationships with clinical parameters. We grouped tumors as high-stiffness (COL1^{High}/LOX^{High}, 32 patients) or low-stiffness (COL1^{Low}/LOX^{Low}, 33 patients) using median

COL1 and LOX expression as thresholds. PFKFB3 protein was substantially more abundant in the high-stiffness tumors (**Figure 3i**).

We then divided patients into PFKFB3-high (n = 43) and PFKFB3-low (n = 44) groups based on median expression. High PFKFB3 was strongly associated with larger tumor diameters, elevated COL1 and LOX, and the stiff-matrix signature (**Table 1**). These correlations link increased PFKFB3 to both disease progression and a mechanically rigid tumor environment, mirroring mechanistic insights from model systems.

Furthermore, patients with high tumoral PFKFB3 expression had significantly shorter overall and disease-free survival. Both univariate and multivariate Cox regression analyses identified high PFKFB3 as an independent predictor of inferior overall survival (**Table 2**) and disease-free survival (**Table 3**) in this HCC cohort.

Table 2. Univariate and multivariate COX regression analysis of clinicopathological features associated with overall survival in HCC patients.

Clinicopathological factors	Overall survival			
	Univariate		Multivariate	
	P-value	HR (95%CI)	P-value	HR (95%CI)
Gender: female vs male	0.015	2.3 (1.2-4.5)	0.018	2.3 (1.2-4.5)
Age(years): > 50 vs ≤ 50	0.100	1.6 (0.91-2.9)	NA	
HBV infection: Positive vs Negative	0.520	1.3 (0.6-2.8)	NA	
TB(umol/L): > 17.1 vs ≤ 17.1	0.840	0.92 (0.41-2.1)	NA	
ALT(U/L): > 40 vs ≤ 40	0.470	0.79 (0.41-1.5)	NA	
PT(s): > 14 vs ≤ 14	0.660	1.2 (0.51-2.9)	NA	
AFP(ng/mL): > 400 vs ≤ 400	0.280	1.4 (0.76-2.5)	NA	
Tumor size(cm): > 5 vs ≤ 5	0.004	2.5 (1.3-4.6)	0.680	1.2 (0.56-2.5)
Tumor number: Multiple vs Solitary	0.089	1.9 (0.9-4.2)	NA	
Vascular invasion: Yes vs No	0.002	2.6 (1.4-4.8)	0.076	1.9 (0.94-3.8)
TNM stage: III vs I-II	0.0004	3.1 (1.7-5.8)	0.019	2.2 (1.1-4.4)
Histological grade: III vs I-II	0.400	1.3 (0.69-2.5)	NA	
PFKFB3: High vs Low	0.0002	3.5 (1.8-6.6)	0.006	2.6 (1.3-5.3)

Abbreviations: PFKFB3 = phosphofructokinase-2/fructose-2,6-bisphosphatase 3; HCC = hepatocellular carcinoma; TB = total bilirubin; ALT = alanine aminotransferase; PT = prothrombin time; HBV = hepatitis B virus; AFP = alpha-fetoprotein; TNM = Tumor Node Metastasis.

Table 3. Univariate and multivariate COX regression analysis of clinicopathological features associated with disease-free survival in HCC patients.

Clinicopathological factors	Disease-free survival			
	Univariate		Multivariate	
	P-value	HR (95%CI)	P-value	HR (95%CI)
Gender: female vs male	0.051	1.9 (1-3.8)	NA	
Age(years): > 50 vs ≤ 50	0.060	1.8 (0.98-3.2)	NA	
HBV infection: Positive vs Negative	0.550	1.3 (0.59-2.7)	NA	
TB(umol/L): > 17.1 vs ≤ 17.1	0.700	0.85 (0.38-1.9)	NA	

ALT(U/L): > 40 vs ≤ 40	0.350	0.74 (0.39-1.4)	NA	
PT(s): > 14 vs ≤ 14	0.490	1.4 (0.57-3.2)	NA	
AFP(ng/mL): > 400 vs ≤ 400	0.400	1.3 (0.71-2.3)	NA	
Tumor size(cm): > 5 vs ≤ 5	0.001	2.8 (1.5-5.2)	0.150	1.7 (0.82-3.6)
Tumor number: Multiple vs Solitary	0.043	2.2 (1-4.8)	0.840	1.1 (0.44-2.7)
Vascular invasion: Yes vs No	0.003	2.4 (1.3-4.4)	0.420	1.3 (0.66-2.7)
TNM stage: III vs I-II	0.001	2.8 (1.5-5.3)	0.097	1.9 (0.89-4)
Histological grade: III vs I-II	0.1	1.7 (0.9-3.3)	NA	
PFKFB3: High vs Low	0.0007	3 (1.6-5.6)	0.014	2.3 (1.2-4.4)

Abbreviations: PFKFB3 = phosphofructokinase-2/fructose-2,6-bisphosphatase 3; HCC = hepatocellular carcinoma; TB = total bilirubin; ALT = alanine aminotransferase; PT = prothrombin time; HBV = hepatitis B virus; AFP = alpha-fetoprotein; TNM = Tumor Node Metastasis.

Radiotherapy and DNA-damaging chemotherapy remain cornerstone non-surgical treatments for many malignancies, yet their effectiveness is frequently limited by the development of resistance [39, 40]. The tumor microenvironment acts as a supportive niche that not only sustains cancer growth and dissemination but also modulates therapeutic sensitivity [41]. Factors such as low oxygen tension, immune evasion, and altered metabolism have been extensively linked to reduced treatment responses [41-47]. In contrast, the influence of physical forces—particularly extracellular matrix stiffness—on chemoradiotherapy resistance and the molecular pathways involved remains poorly characterized. Earlier work from our laboratory showed that increased matrix rigidity enhances the invasive and metastatic potential of HCC cells and contributes to the formation of pre-metastatic niches in the lung [28, 29, 48]. We also observed that stiff matrices can reduce oxaliplatin-induced cell death in HCC [23], suggesting a possible link to DNA repair mechanisms.

Beyond their canonical roles in carbohydrate metabolism, glycolytic enzymes are increasingly recognized for performing additional, non-catalytic functions in cancer cells, including regulation of apoptosis, histone modifications, transcriptional control, and kinase activity [27]. This versatility led us to propose that these enzymes might simultaneously drive proliferation and confer survival advantages under mechanical stress. The present study therefore focused on uncovering how matrix stiffness exploits metabolic enzymes to promote HCC progression and blunt the efficacy of radiotherapy.

A major obstacle in this field has been the scarcity of suitable animal models that allow direct evaluation of matrix stiffness effects on radiation response in vivo. To address this, we established orthotopic HCC models using both irradiated and non-irradiated cancer cells in

livers with either normal or elevated stiffness. Assessment of tumor growth kinetics, Ki-67 proliferation indices, and TUNEL apoptosis staining demonstrated that rigid liver tissue clearly diminishes the effectiveness of radiotherapy by enhancing cancer cell survival after DNA damage. Parallel in vitro experiments using tunable stiffness gels yielded consistent results. Several other reports have similarly noted that higher matrix rigidity decreases sensitivity to various chemotherapeutic agents [49-51]. However, the involvement of non-metabolic functions of glycolytic enzymes in stiffness-mediated radioresistance had not been previously described. Our data also confirmed that harsh conditions accelerate HCC tumor growth and cell division, consistent with prior observations in different rat models [29]. Given the central importance of glycolytic enzymes in sustaining rapid proliferation and cell viability [24, 52], they represent plausible mediators of these dual effects.

Systematic comparison of key glycolytic proteins across stiffness conditions revealed selective and robust induction of PFKFB3, with no significant changes in other major enzymes. This upregulation paralleled increases in glycolytic flux and proliferative activity, both of which were reversed by PFKFB3 depletion. Thus, PFKFB3 appears to be the primary driver of stiffness-enhanced glycolysis and growth in HCC cells. Supporting this notion, elevated PFKFB3 has also been shown to stimulate proliferation in non-malignant tumors [53]. Notably, stiff substrates increased PFKFB3 protein levels without altering its mRNA levels, suggesting post-translational control. We subsequently demonstrated that high stiffness inhibits PFKFB3 ubiquitination and identified NEDD4 as its specific E3 ubiquitin ligase. Further experiments delineated how stiffness downregulates NEDD4 expression, primarily through miR-199a-3p. NEDD4 knockdown experiments confirmed its role in controlling PFKFB3 stability via

ubiquitination. Complementary work in glioblastoma has shown that SUMOylation of PFKFB3 can similarly block its ubiquitination, thereby boosting glycolysis and tumor growth [54], underscoring the broader significance of post-translational modifications in PFKFB3 regulation. DNA double-strand breaks are primarily resolved by homologous recombination (HR) or non-homologous end joining (NHEJ). Prior studies reported that PFKFB3 inhibition elevates γ -H2AX while reducing RAD51 in certain cancers [55, 56], and that PFKFB3 silencing impairs AKT signaling and ERCC1 expression in HCC, compromising repair capacity [57]. More recent evidence indicates that PFKFB3 is rapidly recruited to radiation-induced damage sites and co-localizes with repair factors in the HR pathway [58]. However, its participation in stiffness-dependent repair via NHEJ had not been explored. Using multiple approaches—including subcellular fractionation, tissue immunohistochemistry, and nuclear protein analysis—we showed that high matrix stiffness strongly promotes PFKFB3 nuclear accumulation specifically in irradiated HCC cells. In non-irradiated cells, although total PFKFB3 is higher under stiff conditions, the majority remains cytoplasmic, consistent with its primary metabolic role; only a modest nuclear fraction is present, potentially influencing other nuclear functions such as cell cycle regulation [59]. Radiation combined with stiff matrices synergistically drives pronounced nuclear translocation. The absence of stiffness-dependent repair differences without irradiation likely reflects insufficient DNA damage to fully engage this nuclear function.

Pharmacologic inhibition of importin β with importazole reduced both nuclear PFKFB3 entry and DNA repair efficiency on stiff substrates, reinforcing the importance of nuclear translocation. Site-directed mutagenesis of the PFKFB3-Ku70 interface further implicated the NHEJ pathway in stiffness-augmented repair.

Although PFKFB3 inhibitors have shown anti-cancer activity in preclinical models [60, 61], their clinical translation faces hurdles. Systemic blockade could interfere with normal glucose metabolism and physiological processes. Moreover, enzymatic inhibition may not fully prevent nuclear translocation or overcome radioresistance. Targeting NEDD4 presents additional challenges due to its multiple substrates, requiring careful future evaluation.

Several limitations should be acknowledged. While we provide comprehensive mechanistic insights into stiffness-induced PFKFB3 upregulation, we have not yet

performed direct in vivo functional manipulation of PFKFB3 in animal models. Additionally, although the PFKFB3-Ku70 interaction clearly contributes to NHEJ-mediated repair under stiff conditions, contributions from other repair pathways cannot be ruled out entirely.

In summary, this study identifies a mechanosensitive pathway centered on PFKFB3 that drives HCC progression and confers radiotherapy resistance (**Figure 7**). Specifically, matrix stiffness downregulates NEDD4, thereby reducing PFKFB3 ubiquitination, stabilizing the protein, enhancing glycolysis, and promoting tumor growth and proliferation (**Figure 7**). Concurrently, stiffness facilitates PFKFB3 nuclear translocation in irradiated cells, where it interacts with Ku70 to bolster NHEJ-dependent DNA repair, ultimately diminishing treatment efficacy (**Figure 7**).

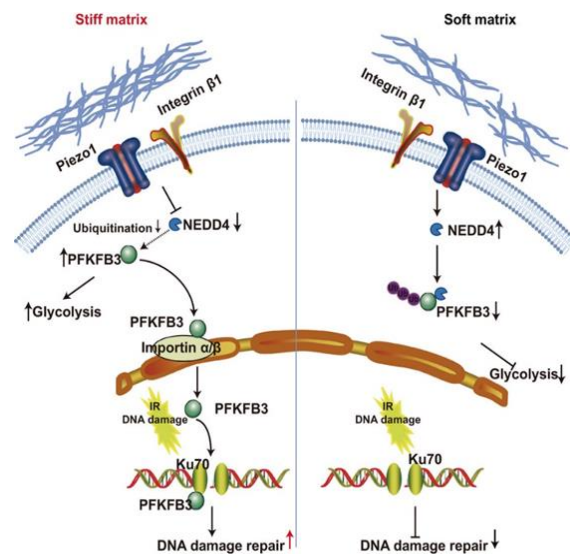


Figure 7. Schematic overview of the mechanism by which matrix stiffness regulates glycolysis and DNA repair in hepatocellular carcinoma.

Elevated matrix rigidity downregulates the E3 ubiquitin ligase NEDD4, thereby reducing PFKFB3 ubiquitination. This leads to greater PFKFB3 protein stability, elevated glycolytic activity, and enhanced tumor cell proliferation. In parallel, stiff matrix conditions promote nuclear translocation of PFKFB3 in irradiated HCC cells, where it binds to Ku70 and augments DNA damage repair capacity.

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Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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