Published in final edited form as:
Hepatology. 2013 July ; 58(1): 182–191. doi:10.1002/hep.26310.

TARDBP regulates glycolysis in hepatocellular carcinoma by regulating PFKP through miR-520

Yun-Yong Park[1], **Sang Bae Kim**[1], **Hee Dong Han**[2,6], **Bo-Hwa Sohn**[1], **Ji-Hoon Kim**[1,7], **Jiyong Liang**[1], **Yiling Lu**[1], **Gordon B. Mills**[1], **Anil K. Sood**[2,3,4,5], and **Ju-Seog Lee**[1,8]

[1]Department of Systems Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

[2]Department of Gynecologic Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

[3]Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

[4]Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

[5]Center for RNA Interference and Non-Coding RNA, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

[6]Research Center for Medicinal Chemistry, Division of Drug Discovery Research, Korea Research Institute of Chemical Technology, 141 Gajeong-ro, Yuseong-gu, Deajeon 305-600, South Korea

[7]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Korea University School of Medicine, Seoul, Korea

[8]Department of Biochemistry and Molecular Biology, Medical Research Center and Biomedical Science Institute, School of Medicine, Kyung Hee University, Seoul 130-701, Korea

Abstract

Metabolic changes are common features of many cancer cells and are frequently associated with the clinical outcome of patients with various cancers including hepatocellular carcinoma (HCC). Thus, aberrant metabolic pathways in cancer cells are attractive targets for cancer therapy. However, our understanding of cancer-specific regulatory mechanisms of cell metabolism is still very limited. We found that Tat activating regulatory DNA-binding protein (TARDBP) is a novel regulator of glycolysis in HCC cells. TARDBP regulates expression of the platelet isoform of phosphofructokinase (PFKP), the rate-limiting enzyme of glycolysis that catalyzes the irreversible conversion of fructose-6-phosphate to fructose-1,6-bisphosphate. Silencing of TARDBP expression in multiple HCC cell lines leads to impaired glucose metabolism and inhibition of *in vitro* and *in vivo* growth of HCC cells. Notably, the miR-520 family is an intermediate regulator of TARDBP-mediated regulation of glycolysis. Mechanistically, TARDBP suppressed expression of the miR-520 family, which in turn inhibited expression of PFKP. We further showed that expression of TARDBP is significantly associated with the overall survival of patients with HCC.

Conclusion—Our study provides new mechanistic insights into the regulation of glycolysis in HCC cells and reveals TARDBP as a potential therapeutic target for HCC.

Competing financial interests

The authors declare no competing financial interests.

Supporting Information is available at Hepatology Online.

Subject terms

TARDBP; PFKP; Glycolysis; miR-520; Hepatocellular carcinoma

INTRODUCTION

TARDBP was identified first as a transcription factor that binds to the human immunodeficiency virus transactivation response region[1] and later as an RNA-binding protein linked to neurodegenerative diseases such as frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS).[2–4] *TARDBP* is one of frequently mutated genes in sporadic and familial ALS, as well as in patients with FTLD, providing evidence of a direct link between *TARDBP* abnormalities and neurodegeneration.[4] Although roles of TARDBP have been extensively studied in motor neuron linking to FTLD and ALS, recent reports suggested that TARDBP might play important roles in cellular metabolisms including glucose metabolism and lipid metabolism.[5, 6] In addition, recent studies also suggested functional roles of *TARDBP* in human cancer.[7–9] *TARDBP* expression is significantly altered in leukemia and TARDBP is significantly associated with susceptibility to Ewing sarcoma.[8, 9] However, while link of TARDBP in human diseases have been confirmed by numerous reports, it is not clear how TARDBP contributes to diseases because very little is known about the molecular functions of TARDBP except for its roles in RNA metabolism.[10]

Most cancer cells including hepatocellular carcinoma (HCC) cells have very high demand for cellular metabolism to meet the need for new building blocks and energy required for cell growth.[11–13] In particular, oncogenic transformation of cells is frequently associated with an increase in glycolytic flux, mainly caused by increased expression of glycolysis-regulating genes. *MYC* and *HIF1A* are best known transcriptional regulators controlling expression of glycolysis genes such as *LDHA*, *HK2*, *PDK1* and *GLUT1*, whose expression levels are highly elevated in cancer cells.[14, 15] However, because glycolysis is highly facilitated in cancer cells, more transcriptional regulators that actively promote glycolysis are expected to be involved.

In this study, we demonstrated that expression of *TARDBP* is significantly elevated in HCC and that it regulates expression of *PFKP*, rate-limiting enzyme for glycolysis, through negative-regulation of miR-520s. Thus, our study provides evidence that *TARDBP* is a novel transcriptional regulator of glycolysis in cancer and potential therapeutic target for treatment of patients with HCC.

Materials and methods

Additional Materials and Methods information can be found in the online Supporting Information (Supporting Materials).

shRNA, siRNA and miRNA mimics

shTARDBP (#38-TRCN0000016038 and #40-TRCN0000016040) and shControl (SHC002) clones were purchased from Sigma. The cell lines of interest were then infected with virus particles from the 293 cells. Virus-infected cells were selected for 3 days with 2μg/ml puromycin. siLuc, siTARDBP (SMARTpool)[16] and miRNA mimics were purchased from Dharmacon. Cells were transfected with indicated siRNA or mimic-miRNA using Oligofectamine (Invitrogen) for 72 hrs and used for assays.

Detection of glucose uptake, lactate levels and cellular ATP levels

Glucose uptake and lactate level were measured using a Multiparameter Bioanalytical System (#YSI 7100; YSI Life Sciences). After SK-Hep1 cells were transduced with shRNA viruses, the media from cultured cells were used for measuring glucose or lactate levels. Amount of glucose taken up by cancer cells were measured by subtraction of remained glucose level in cultured media from total glucose level in uncultured media. Cellular ATP level was determined using an ATP Colorimetric Assay Kit (#K354-100; BioVision) and normalized to cell number.

Xenograft experiments

Female athymic nude mice *(NCr-nu)* were purchased from the NCI-Frederick Cancer Research and Development Center (Frederick, MD) and maintained as previously described.[17] Tumor implantation, siRNA incorporation into dioleoyl phosphatidylcholine (DOPC) nanoliposomes and delivery *in vivo* were carried out as previously described.[17, 18] For TARDBP silencing experiments, mice were randomized into one of the following treatment groups (n = 10 per group): control siRNA-DOPC (150 μg/kg i.v. twice weekly) and TARDBP siRNA-DOPC (150 μg/kg i.v. twice weekly). The control siRNA sequence used was UUCUCCGAACGUGUCACGU [dT][dT], and TARDBP siRNA sequences were the same as those used for the cell line experiments. Human HCC cells (SK-Hep1) were subcutaneously injected into mice (1.0×10^6 cells/animal) on day 0 and siRNA treatment was started on day 7. Five weeks later, mice were euthanized and subjected to necropsy, and tumors were harvested.

Results

TARDBP expression is elevated in HCC

Because recent studies suggested a potential link of *TARDBP* to cancer, we first examined the expression level of *TARDBP* in human liver tissues. Expression of *TARDBP* was significantly higher in tumors than in normal liver tissues surrounding the tumors ($P = 1.0 \times 10^{-14}$ by Student's t-test, Fig. 1A), indicating potential roles of *TARDBP* in HCC. Consistent with the gene expression data from patient tissues, expression of *TARDBP* was detected in all HCC cell lines examined (Fig. 1B).

We next depleted expression of *TARDBP* with specific siRNAs to *TARDBP* to test whether *TARDBP* plays significant roles in the growth of HCC cells. Silencing of *TARDBP* expression with specific siRNAs significantly attenuated growth of SK-Hep1 and HUH7 cells (Fig. 1C and Supporting Fig. 1A), strongly suggesting that *TARDBP* is necessary for growth and survival of HCC cells. Consistent with cell growth assay, the colony formation is also significantly reduced upon depletion of TARDBP with specific siRNAs (Fig. 1D). Similar levels of growth inhibition upon silencing of *TARDBP* expression were observed in additional HCC cells (SNU-449 and Hep3B) (Supporting Fig. 1A and B).

In agreement with previous reports,[1, 2] cell fractionation showed that TARDBP is predominantly localized in the nucleus of SK-Hep1 cells (Fig 1E and Supporting Fig. 1C), suggesting that its biological roles in cancer cell growth might be mediated by its roles as a transcription factor or regulator of RNA processing.

TARDBP regulates glycolysis in HCC cells

To investigate downstream targets of *TARDBP* that could regulate cell growth, we carried out microarray experiments after depleting *TARDBP* in SK-Hep1 cells (Fig. 2A). As expected, silencing of *TARDBP* expression led to down-regulation of genes involved in cell growth (i.e, *CDK6*, *RANBP1* and *CENPE*). Surprisingly, a large number of the down-

regulated genes are directly involved in glucose transport and glycolysis (i.e., *SLC2A1*, *PFKP*, *PFKFB4*, *PGK1* and *ENO2*), strongly suggesting potential roles of *TARDBP* in regulating glucose metabolism. Notably, expression of *PFKP*, among many glycolysis-related genes, was most significantly altered by *TARDBP* ($P = 7.5 \times 10^{-5}$ by Student's *t*-test, 4.7-fold). Expression of *PFKP* and other glycolysis-related genes was also significantly down-regulated by depleting *TARDBP* in two additional HCC cell lines (FOCUS and HUH7) when their expression was assessed by quantitative real-time polymerase chain reaction (qRT-PCR) (Fig. 2B and C). These results strongly suggested conserved and universal roles of *TARDBP* in glucose metabolism in HCC cells, particularly through regulation of *PFKP*.

Phosphofructokinase (PFK) is a key regulatory enzyme in glycolysis that catalyzes the irreversible conversion of fructose-6-phosphate to fructose-1,6-bisphosphate. Humans have three PFK isoforms: liver (*PFKL*), muscle (*PFKM*) and platelet (*PFKP*).[19, 20] Thus, we examined whether *TARDBP* regulates expression of the other PFK isoforms in addition to *PFKP*. The results showed that protein expression of PFKL and PFKM was not altered by silencing of *TARDBP* expression, while all three siRNAs specific to *TARDBP* successfully down-regulated expression of PFKP in SK-Hep1 cells (Fig. 2D). Consistent with western blot experiments, qRT-PCR experiments showed that *TARDBP* regulates expression of only *PFKP* in SK-Hep1 cells (Fig. 2E).

Since *TARBDP* regulated expression of many glycolysis genes including *PFKP* in multiple HCC cells, we determined effect of depletion of TARDBP in metabolic response. Glucose uptake of SK-Hep1 cells was significantly reduced by silencing of *TARDBP* expression (Fig. 3A and B). Furthermore, silencing of *TARDBP* expression resulted in a decrease in lactate production and ATP levels indicating a decrease of glycolysis (Fig. 3B). Thus, our findings strongly support the proposed roles of *TARDBP* in HCC cell growth through regulation of glucose and energy metabolism.

miR-520s down-regulates PFKP expression

We next attempted to determine the molecular mechanism of how *TARDBP* regulates *PFKP* expression. Given that the best known function of TARDBP is RNA processing as an RNA-binding protein,[21] we examined whether TARDBP directly interacts with mRNA of *PFKP*. However, analysis of RNA immunoprecipitation data with anti-TARDBP antibody [21] failed to demonstrate interaction of TARDBP with *PFKP* mRNA (Supporting Fig. 2), suggesting that *TARDBP* likely regulates *PFKP* by other mechanisms. Because *TARDBP* positively regulates expression of *PFKP* and also functions as a transcription repressor,[22] we hypothesized that *PFKP* could be negatively regulated by intermediate regulators that are in turn directly suppressed by *TARDBP*. Recent studies showed that *TARDBP* is involved in regulation of miRNAs,[23, 24] suggesting that miRNAs might be good candidates for intermediaries between *TARDBP* and *PFKP*. To identify such intermediary regulators, we explored target miRNAs that can suppress *PFKP* based on sequence alignment (Fig. 4A). Sequence analysis with the starBase database[25] revealed that 26 miRNAs contain direct binding sequences for the *PFKP* 3′UTR (Supporting Table 1). Interestingly, three all independent prediction programs (target Scan, picTar and miRanda) predicted miR-520 and miR-302 family as major regulatory miRNAs for *PFKP*.[26] Because previous studies showed that miR-520b and miR-520e can inhibit cancer cell growth,[27–29] we next tested if inhibition of cell growth by miR-520 is mediated by regulation of *PFKP* expression. When SK-Hep1 cells were treated miR-520a-3p, miR-520b, and miR-520e (here after miR-520a/b/e), expression of *PFKP* was significantly down-regulated (Fig. 4B), suggesting that *PFKP* might be a direct target of miR-520a/b/e. However, expression of other glycolysis genes were not significantly altered by miR-520a/b/e (Supporting Fig. 3), suggesting that these

miRNAs regulate glycolysis mainly through inhibition of PFKP. To determine whether miR-520a/b/e directly targets the 3′UTR of *PFKP* mRNA, we assessed a luciferase reporter vector containing the 3′UTR sequence of *PFKP*, including the predicted binding site for miR-520a/b/e in SK-Hep1 cells. Luciferase activity was significantly inhibited by the *PFKP* 3′UTR sequence when only miR-520a/b/e were co-transfected (Fig. 4C). However, luciferase activity was not inhibited by a mutant 3′UTR sequence (Fig. 4D and E), strongly demonstrating that miR-520a/b/e directly targets the 3′UTR sequence of *PFKP* mRNA and inhibits the expression of *PFKP*.

TARDBP regulates PFKP by suppressing miR-520s

Since expression of *PFKP* was down-modulated by miR-520s, we next tested whether miR-520a/b/e are regulated by *TARDBP* by measuring expression of these miRNAs by qRT-PCR after silencing of TARDBP in SK-Hep1 and SNU449. The results showed that expression of miR-520a/b/e was significantly increased when *TARDBP* was silenced (Fig 5A), with expression of miR-520b strongly induced by silencing *TARDBP* in two HCC cell lines. TARDBP is a DNA-binding protein that binds to the GTGTGT sequence in target promoter regions.[22] Thus, we examined whether TARDBP can directly bind to the *miR-520b* promoter. Indeed, a chromatin immunoprecipitation (ChIP) assay demonstrated that TARDBP directly bound to the *miR-520b* promoter region, specifically in GT-rich regions (Fig 5B), suggesting that miR-520b is indeed a direct downstream target of *TARDBP*.

Our results strongly suggested that growth inhibition following depletion of TARDBP might be mediated by miR-520a/b/e. To test this hypothesis, we introduced miR-520a/b/e into two HCC cell lines and observed that cell growth was significantly reduced (Supporting Fig. 4). To further test whether growth inhibition is mediated by down-regulation of *PFKP*, we introduced exogenous myc-tagged *PFKP* after silencing *TARDBP* in SK-Hep1 cells. Because myc-tagged *PFKP* lacks 3′ UTR sequence, its expression is not inhibited by miR-520s (Fig. 5C). Growth inhibition by silencing *TARDBP* was rescued by exogenous *PFKP* (Fig. 5D). Furthermore, glucose uptake, lactate production, and ATP level were significantly increased by exogenous *PFKP* (Fig. 5E). When induced miR-520b in *TARDBP*-silenced SK-Hep1 was inhibited by specific antisense miR-520b inhibitor, expression of *PFKP* is recovered (Supporting Fig. 5), demonstrating that regulation of *PFKP* by *TARDBP* is mediated through miR-520s. Taken together, our data suggested that *PFKP* is main regulatory target for *TARDBP*-miR-520s mediated regulation of cell growth. These observations agree very well with the finding of previous studies showing that miR-520b and miR-520e might function as negative regulators of cell proliferation.[27, 30]

Clinical relevance of TARDBP, PFKP, and miR-520s expression in cancer

Our data suggested functional roles of TARDBP and PFKP as positive regulators and miR-520s as negative regulator of cell proliferation. This view is strongly supported by expression patterns of these genes in the NCI-60 cancer cell lines. Expression of both *TARDBP* and *PFKP* was very high in the vast majority of the 60 cancer cell lines (Supporting Fig. 6A). In contrast, *miR-520a/b/e* were barely expressed, strongly supporting its role as negative regulator of cell growth. Expression of *PFKP* was the highest among PFK isoforms in NCI-60 cell lines (Supporting Fig. 6B), further supporting that cancer-specific expression of *PFKP* is regulated by *miR-520a/b/e* and *TARDBP*.

We next assessed the clinical relevance of *TARDBP* in HCC. Expression of *TARDBP* is significantly associated with prognosis when estimated by receiver operating characteristic analysis. Areas under curve of *TARDBP* expression over 3-year overall survival is 0.6 (95% CI, 0.53 – 0.66, $P = 0.007$) (Fig. 6A). When patients were stratified according to expression

level of *TARDBP*, patients with high TARDBP expression showed significantly shorter survival ($P = 3.8 \times 10^{-4}$, Fig. 6B). Association of *TARDBP* with prognosis is further supported by its significant correlation with 65-gene risk score ($r = 0.5$; $P = 2.2 \times 10^{-16}$) (Fig. 6C) that was previously developed for prediction of recurrence.[31] Significant positive correlation between expression of *TARDBP* and *PFKP* in HCC patients is also concordant with their roles as positive regulator for cell growth (Fig. 6D).

Therapeutic efficacy of PFKP in a mouse model

The critical roles of *TARDBP* and its downstream targets, the miR-520 family, in cell growth and the significant correlation of *TARDBP* with patient survival strongly suggested that TARDBP and its downstream targets would be potential therapeutic targets for cancer treatment. To test this, we carried out a mouse xenograft experiment with SK-Hep1 cells and siRNA specific to *TARDBP*. Compared with treatment with control siRNA, treatment with siTARDBP resulted in significant reduction in tumor weight (Fig. 7A), recapitulating the effects of silencing TARDBP *in vitro*. Efficient silencing of *TARDBP* by siRNA was confirmed by immunostaining of TARDBP and its downstream target PFKP and further validated by qRT-PCR (Fig. 7B and C and Supporting Fig. 7). As expected, cell proliferation, as examined by Ki67 immunostaining, was significantly decreased in tumors treated with siTARDBP (Fig. 7B). In addition, lactate and ATP levels were also significantly decreased (Fig. 7C) and expression of miR-520b and miR-520e (Fig. 7D) was significantly increased in siTARDBP treated mice compared to control. These results clearly demonstrate the importance of *TARDBP* in tumor growth and the potential of *TARDBP* as a therapeutic target.

DISCUSSION

In the current work, we have presented a mechanistic link from *TARDBP* to *PFKP*, the rate-limiting enzyme of glycolysis, and we also have provided evidence suggesting that this pathway is associated with poor prognosis of HCC. A notable finding was the identification of the miR-520 family as an intermediary regulator of this pathway.

While *TARDBP* was originally identified as a transcription repressor binding to the HIV transactivation response region,[1] down-stream targets and molecular mechanisms related to its transcription repressor activity has not been properly explored. Our data demonstrated that *TARDBP* function as a transcription repressor and its molecular activity as transcription repressor plays key roles in regulation of glycolysis in cancer cells. *TARDBP* strongly represses expression of miR-520 family as evidenced by significant increase in their intracellular levels upon *TARDBP* depletion. This notion was strengthen by a ChIP assay demonstrating that *TARDBP* directly bound to GT-rich regions in the *miR-520b* promoter. In addition to regulation of miR-520s in transcriptional level, *TARDBP* may regulate miR-520s post-transcriptionally because the involvement of *TARDBP* in miRNA processing has been observed in other systems.[23, 24] Our data are in good agreement with previous studies demonstrating repression of the spermatid-specific gene SP-10 expression by *TARDBP*.[22, 32] Thus, our study rediscovered previously recognized molecular function of *TARDBP* and updated molecular mechanisms and biological roles of *TARDBP* especially in cellular metabolism with use of miRNAs as intermediary regulators.

There are growing evidences supporting that miRNAs play important roles in regulation of cellular metabolism.[33, 34] Recent studies revealed that miRNAs such as miR-124, miR-137, miR-340 miR-143 and miR-155 regulate glycolysis by directly targeting the 3′ UTR region of *HK2* and *PKM2*.[35–37] With prediction analysis based on sequence, we discovered that miR-520a/b/e are major regulators of glycolysis by directly targeting the 3′UTR of *PFKP*

mRNA. We further demonstrated that *TARDBP*-mediated suppression of miR-520a/b/e is important for growth and survival of HCC cells.

Importantly, analyses of gene expression patterns from multiple cancer lineages provide evidences supporting our findings related to molecular functions and mechanisms of *TARDBP*-mediated *PFKP* regulation. Expression of *TARDBP* is significantly higher in tumors than normal tissues. We also have found inversed correlation of expression patterns among *TARDBP*, *PFKP* and miR-520s. Expression of *TARDBP* and *PFKP* is significantly high in vast majority of cancer cell lines while expression of miR-520s is very low (Supporting Fig. 4A). This is in good agreement with mechanism postulating that *TARDBP* suppresses expression of miR-520s that directly inhibit *PFKP* to maintain increased glycolysis in cancer cells (Fig. 7E).

In conclusion, we found that novel roles of *TARDBP* linked to glycolysis via *PFKP* in HCC. Deregulation of cellular metabolism is one of the hallmarks of cancer cells,[38] and altered components of the metabolic pathway represent attractive therapeutic targets.[13, 39] Thus, the identification of the *TARDBP*-miR-520-*PFKP* axis regulating glycolysis and ATP production elicits a potential new approach to target the tumor-specific metabolic pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research is supported in part by 2011 and 2012 cycle of MD Anderson Sister Institute Network Fund (J-S.L.), 5U54 CA112970-08 (G.B.M.), 5P01CA099031-07 (G.B.M.), P30 CA016672 (G.B.M.), and CA016672 (MD Anderson Cancer Center Support Grant) from National Institutes of Health

References

1. Ou SH, Wu F, Harrich D, Garcia-Martinez LF, Gaynor RB. Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type 1 TAR DNA sequence motifs. J Virol. 1995; 69:3584–3596. [PubMed: 7745706]
2. Buratti E, Dork T, Zuccato E, Pagani F, Romano M, Baralle FE. Nuclear factor TDP-43 and SR proteins promote in vitro and in vivo CFTR exon 9 skipping. EMBO J. 2001; 20:1774–1784. [PubMed: 11285240]
3. Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, Bruce J, et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science. 2006; 314:130–133. [PubMed: 17023659]
4. Lee EB, Lee VM, Trojanowski JQ. Gains or losses: molecular mechanisms of TDP43-mediated neurodegeneration. Nat Rev Neurosci. 2012; 13:38–50. [PubMed: 22127299]
5. Tauffenberger A, Vaccaro A, Aulas A, Velde CV, Parker JA. Glucose delays age-dependent proteotoxicity. Aging Cell. 2012
6. Chiang PM, Ling J, Jeong YH, Price DL, Aja SM, Wong PC. Deletion of TDP-43 down-regulates Tbc1d1, a gene linked to obesity, and alters body fat metabolism. Proc Natl Acad Sci U S A. 2010; 107:16320–16324. [PubMed: 20660762]
7. Ayala YM, Misteli T, Baralle FE. TDP-43 regulates retinoblastoma protein phosphorylation through the repression of cyclin-dependent kinase 6 expression. Proc Natl Acad Sci U S A. 2008; 105:3785–3789. [PubMed: 18305152]
8. Teittinen KJ, Karkkainen P, Salonen J, Ronnholm G, Korkeamaki H, Vihinen M, Kalkkinen N, et al. Nucleolar proteins with altered expression in leukemic cell lines. Leuk Res. 2012; 36:232–236. [PubMed: 21783252]

9. Postel-Vinay S, Veron AS, Tirode F, Pierron G, Reynaud S, Kovar H, Oberlin O, et al. Common variants near TARDBP and EGR2 are associated with susceptibility to Ewing sarcoma. Nat Genet. 2012; 44:323–327. [PubMed: 22327514]
10. Chen-Plotkin AS, Lee VM, Trojanowski JQ. TAR DNA-binding protein 43 in neurodegenerative disease. Nat Rev Neurol. 2010; 6:211–220. [PubMed: 20234357]
11. Warburg O. On the origin of cancer cells. Science. 1956; 123:309–314. [PubMed: 13298683]
12. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science. 2009; 324:1029–1033. [PubMed: 19460998]
13. Vander Heiden MG. Targeting cancer metabolism: a therapeutic window opens. Nat Rev Drug Discov. 2011; 10:671–684. [PubMed: 21878982]
14. Dang CV, Le A, Gao P. MYC-induced cancer cell energy metabolism and therapeutic opportunities. Clin Cancer Res. 2009; 15:6479–6483. [PubMed: 19861459]
15. Kim JW, Gao P, Liu YC, Semenza GL, Dang CV. Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. Mol Cell Biol. 2007; 27:7381–7393. [PubMed: 17785433]
16. Fiesel FC, Voigt A, Weber SS, Van den Haute C, Waldenmaier A, Gorner K, Walter M, et al. Knockdown of transactive response DNA-binding protein (TDP-43) downregulates histone deacetylase 6. EMBO J. 2010; 29:209–221. [PubMed: 19910924]
17. Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, Jennings NB, et al. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. Nat Med. 2006; 12:939–944. [PubMed: 16862152]
18. Park YY, Jung SY, Jennings N, Rodriguez-Aguayo C, Peng G, Lee SR, Kim SB, et al. FOXM1 mediates Dox resistance in breast cancer by enhancing DNA repair. Carcinogenesis. 2012
19. Sanchez-Martinez C, Estevez AM, Aragon JJ. Phosphofructokinase C isozyme from ascites tumor cells: cloning, expression, and properties. Biochem Biophys Res Commun. 2000; 271:635–640. [PubMed: 10814514]
20. Sola-Penna M, Da Silva D, Coelho WS, Marinho-Carvalho MM, Zancan P. Regulation of mammalian muscle type 6-phosphofructo-1-kinase and its implication for the control of the metabolism. IUBMB Life. 2010; 62:791–796. [PubMed: 21117169]
21. Colombrita C, Onesto E, Megiorni F, Pizzuti A, Baralle FE, Buratti E, Silani V, et al. TDP-43 and FUS RNA-binding proteins bind distinct sets of cytoplasmic messenger RNAs and differently regulate their post-transcriptional fate in motoneuron-like cells. J Biol Chem. 2012; 287:15635–15647. [PubMed: 22427648]
22. Lalmansingh AS, Urekar CJ, Reddi PP. TDP-43 is a transcriptional repressor: the testis-specific mouse acrv1 gene is a TDP-43 target in vivo. J Biol Chem. 2011; 286:10970–10982. [PubMed: 21252238]
23. Buratti E, Baralle FE. The multiple roles of TDP-43 in pre-mRNA processing and gene expression regulation. RNA Biol. 2010; 7:420–429. [PubMed: 20639693]
24. Buratti E, De Conti L, Stuani C, Romano M, Baralle M, Baralle F. Nuclear factor TDP-43 can affect selected microRNA levels. FEBS J. 2010; 277:2268–2281. [PubMed: 20423455]
25. Yang JH, Li JH, Shao P, Zhou H, Chen YQ, Qu LH. starBase: a database for exploring microRNA-mRNA interaction maps from Argonaute CLIP-Seq and Degradome-Seq data. Nucleic Acids Res. 2011; 39:D202–209. [PubMed: 21037263]
26. Guo L, Lu Z. Global expression analysis of miRNA gene cluster and family based on isomiRs from deep sequencing data. Comput Biol Chem. 2010; 34:165–171. [PubMed: 20619743]
27. Li BA. A novel tumor suppressor miRNA miR-520e contributes to suppression of hepatoma. Acta Pharmacol Sin. 2012; 33:3–4. [PubMed: 22212428]
28. Zhang S, Shan C, Kong G, Du Y, Ye L, Zhang X. MicroRNA-520e suppresses growth of hepatoma cells by targeting the NF-kappaB-inducing kinase (NIK). Oncogene. 2012; 31:3607–3620. [PubMed: 22105365]
29. Cui W, Zhang Y, Hu N, Shan C, Zhang S, Zhang W, Zhang X, et al. miRNA-520b and miR-520e sensitize breast cancer cells to complement attack via directly targeting 3′UTR of CD46. Cancer Biol Ther. 2010; 10:232–241. [PubMed: 20574151]

30. Hu N, Zhang J, Cui W, Kong G, Zhang S, Yue L, Bai X, et al. miR-520b regulates migration of breast cancer cells by targeting hepatitis B X-interacting protein and interleukin-8. J Biol Chem. 2011; 286:13714–13722. [PubMed: 21343296]

31. Kim SM, Leem SH, Chu IS, Park YY, Kim SC, Kim SB, Park ES, et al. Sixty-five gene-based risk score classifier predicts overall survival in hepatocellular carcinoma. Hepatology. 2012; 55:1443–1452. [PubMed: 22105560]

32. Acharya KK, Govind CK, Shore AN, Stoler MH, Reddi PP. cis-requirement for the maintenance of round spermatid-specific transcription. Dev Biol. 2006; 295:781–790. [PubMed: 16730344]

33. Rottiers V, Naar AM. MicroRNAs in metabolism and metabolic disorders. Nat Rev Mol Cell Biol. 2012; 13:239–250. [PubMed: 22436747]

34. Singh PK, Mehla K, Hollingsworth MA, Johnson KR. Regulation of Aerobic Glycolysis by microRNAs in Cancer. Mol Cell Pharmacol. 2011; 3:125–134. [PubMed: 22792411]

35. Jiang S, Zhang LF, Zhang HW, Hu S, Lu MH, Liang S, Li B, et al. A novel miR-155/miR-143 cascade controls glycolysis by regulating hexokinase 2 in breast cancer cells. EMBO J. 2012; 31:1985–1998. [PubMed: 22354042]

36. Peschiaroli A, Giacobbe A, Formosa A, Markert EK, Bongiorno-Borbone L, Levine AJ, Candi E, et al. miR-143 regulates hexokinase 2 expression in cancer cells. Oncogene. 2012

37. Gao P, Sun L, He X, Cao Y, Zhang H. MicroRNAs and the Warburg Effect: New Players in an Old Arena. Curr Gene Ther. 2012; 12:285–291. [PubMed: 22856603]

38. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144:646–674. [PubMed: 21376230]

39. Dang CV, Hamaker M, Sun P, Le A, Gao P. Therapeutic targeting of cancer cell metabolism. J Mol Med (Berl). 2011; 89:205–212. [PubMed: 21301795]

40. Roessler S, Jia HL, Budhu A, Forgues M, Ye QH, Lee JS, Thorgeirsson SS, et al. A unique metastasis gene signature enables prediction of tumor relapse in early-stage hepatocellular carcinoma patients. Cancer Res. 2010; 70:10202–10212. [PubMed: 21159642]

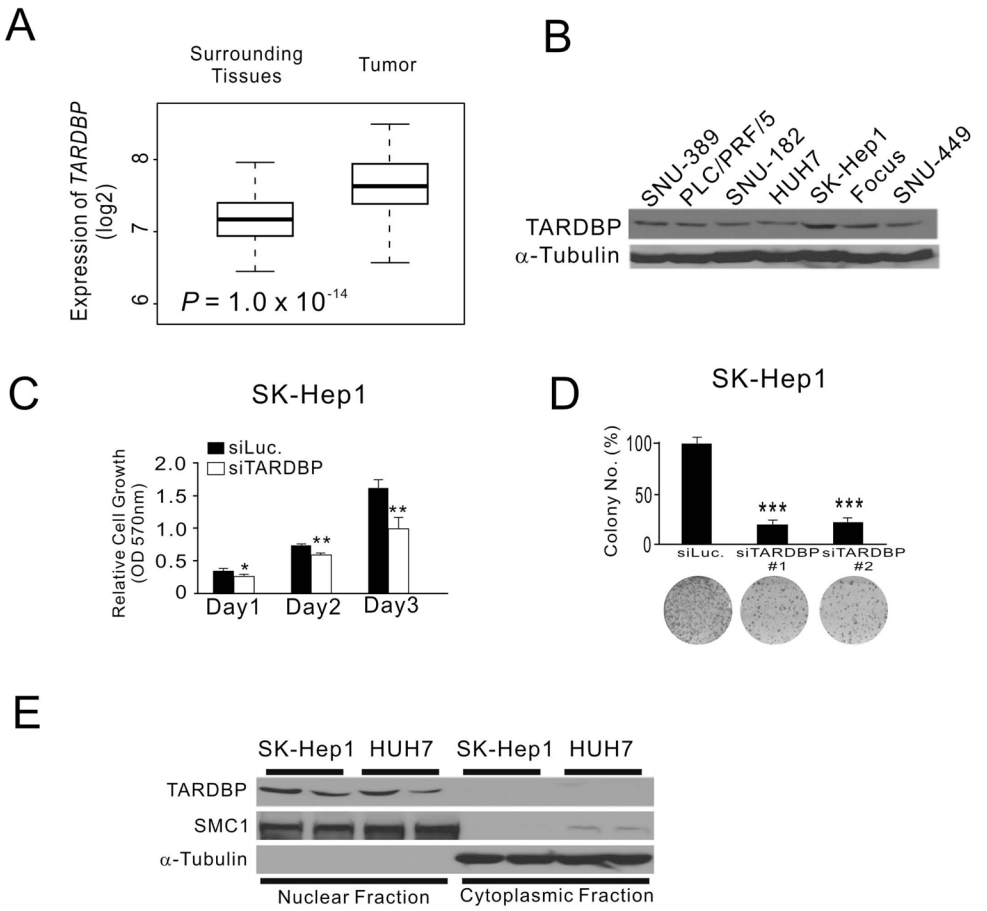

Fig. 1. *TARDBP* expression in HCC cells

(A) Microarray data from Fudan University Liver Cancer Institute (FULCI) cohorts[40] were used to examine expression level of TARDBP in surrounding and tumor tissues. (B) Western blot analysis of 7 HCC cell lines with anti-TARDBP and anti-α-tubulin antibodies. (C, D) MTT and clonogenicity assay after siRNAs transfection in SK-Hep1. (E) Western blot analysis of fractionated cell lysates (nucleus and cytoplasmic parts) from SK-Hep1 and HUH7 with indicated antibodies. Data represent mean ± standard deviation (s.d.) from three independent replicates (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ by Student's *t*-test).

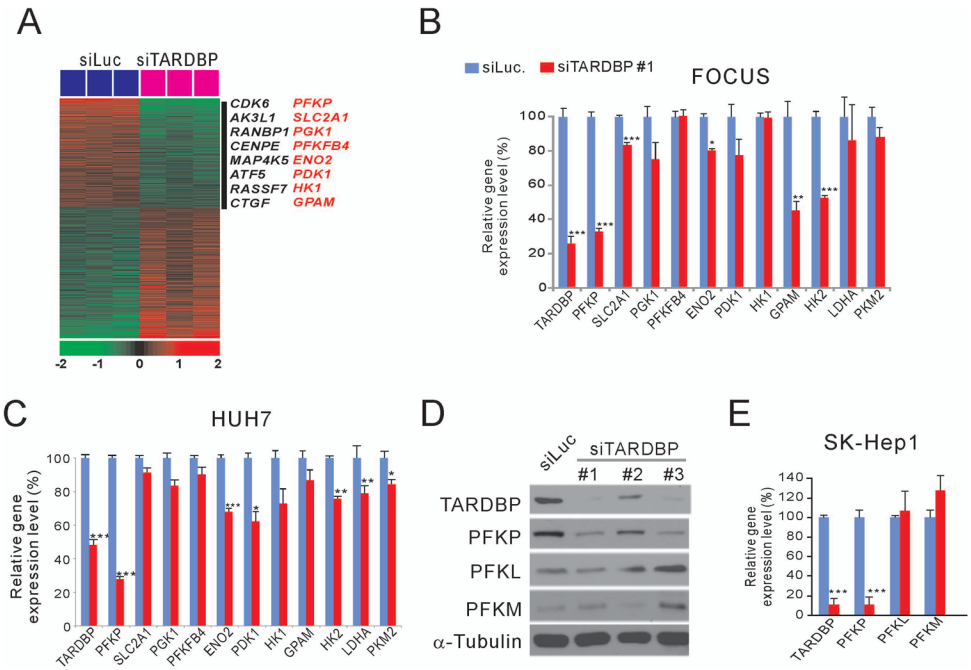

Fig. 2. PFKP expression measurement by silencing TARDBP

(A) Gene expression data from SK-Hep1 cells transfected with siLuc or siTARDBP. A total of 1019 genes were identified as significantly changed by silencing of TARDBP expression ($P < 0.001$). Data are presented in matrix format; each row represents an individual gene, and each column represents a transfected SK-Hep1 cell sample. In the matrix, red and green reflect relatively high and low expression levels of genes, respectively, as indicated in the scale bar (a $\log_2$-transformed scale). Genes involved in glycolysis are highlighted in red text. (B, C) qRT-PCR experiments with mRNAs from the indicated cancer cell lines after transfection with siLuc or siTARDBP. Data represent mean ± s.d. from three independent replicates. (D) Western blot analysis of the three isoforms of the glycolysis enzyme PFK in SK-Hep1 cells transfected with indicated siRNAs. (E) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments with mRNAs from SK-Hep1 cell line after transfection with siLuc or siTARDBP (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ by Student's *t*-test).

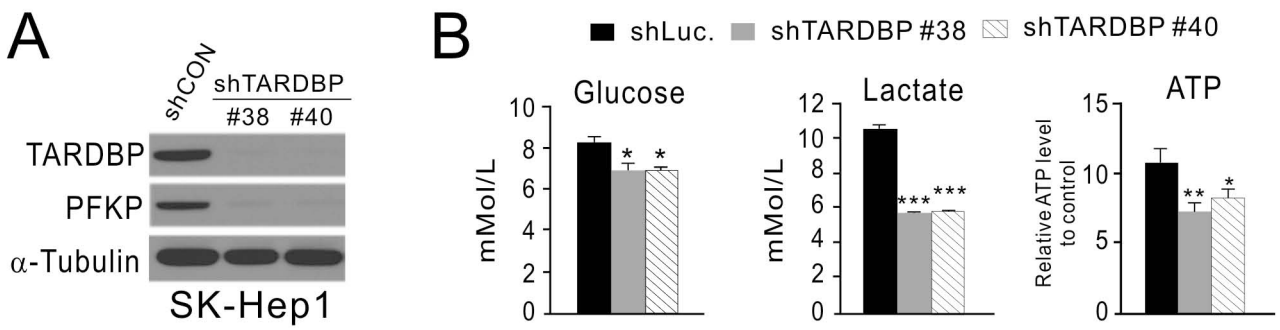

Fig. 3. Glucose metabolite measurement in HCC cell

(A) Western blot analysis for SK-Hep1 cells stably transduced with *TARDBP* (#38 and #40) and control short hairpin RNAs. (B) Analysis of metabolites in SK-Hep1 cells stably transduced with the indicated short hairpin RNAs. Glucose uptake and lactate level were measured from media of cell culture. Intracellular ATP levels were measured at 72 hrs after lentiviral infection. Data represent mean ± s.d. from three independent replicates (**P* < 0.05, ***P* < 0.01, ****P* < 0.005 by Student's *t*-test).

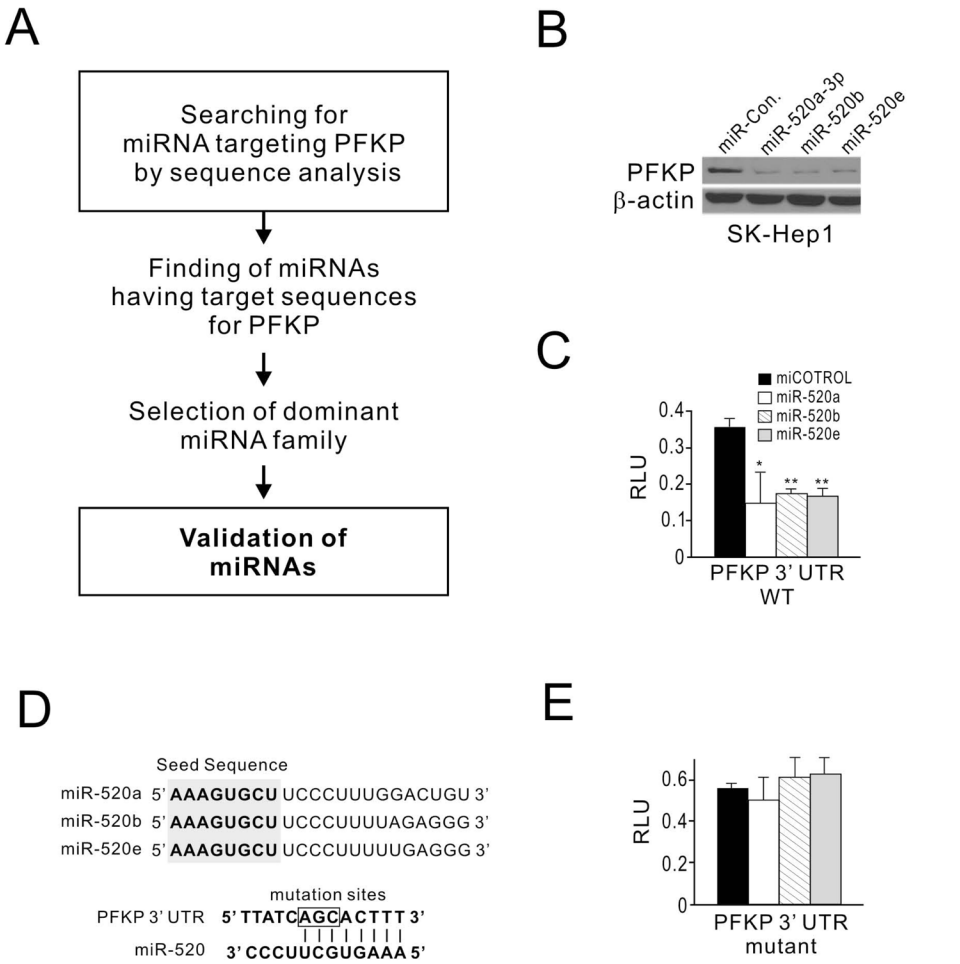

Fig. 4. PFKP gene expression by miR-520

(A) Schematic diagram of strategy for identification miRNAs targeting *PFKP*. (B) Western blot analysis of SK-Hep1 cells after transfecting with miR-520a-3p, miR-520b and miR-520e. β-actin was used as an internal control. (C) Firefly luciferase assay with a luciferase reporter vector containing the wild-type 3′UTR sequence of PFKP in SK-Hep1 cells. Luciferase activities were measured after transfecting the indicated miRNAs. Data represent means ± s.d. from three independent replicates. (D) Sequence alignment within miR-520a/b/e and between miR-520b and the 3′UTR of human *PFKP*. Seed sequences are highlighted in the gray box. (E) Firefly luciferase assay with luciferase reporter vector containing a mutant 3′UTR sequence of *PFKP* in SK-Hep1 cells. Luciferase activities were measured after transfecting the indicated miRNAs (**P* < 0.05, ***P* < 0.01 by Student's *t*-test).

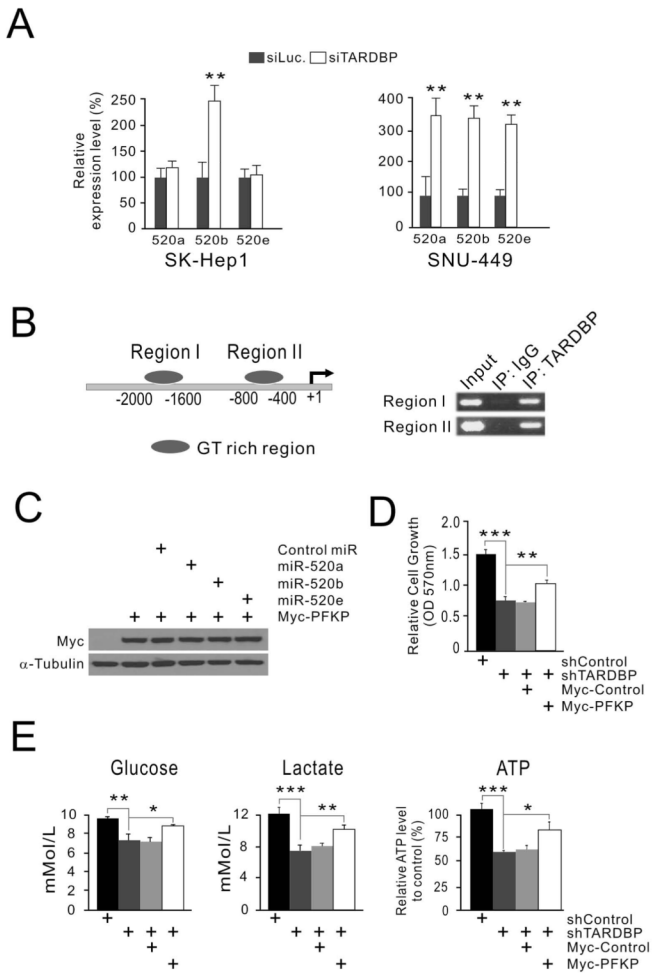

Fig. 5. The miR-520 family is a key intermediate regulator of TARDBP-mediated PFKP expression

(A) qRT-PCR assay with RNAs from the indicated cell lines after transfection with indicated siRNA. Expression of three miRNAs (miR-520a-3p, miR-520b and miR-520e) was measured with specific primers. Data represent mean ± s.d. from three independent replicates (**P* < 0.05, ***P* < 0.01 by Student's *t*-test). (B) ChIP assay with anti-TARDBP antibody in SK-Hep1 cells. The left panel shows a schematic representation of *miR-520b* promoter regions for the ChIP assay. Recruitment of TARDBP to the *miR-520b* promoters was analyzed using primers specific to the indicated promoter region. IgG was used as an internal control. (C) SK-Hep1 cells were transfected with indicated cDNA and miRNA mimics. The cells were used for western blot with indicated antibody. (D, E) After SK-Hep1 were infected with shTARDBP (or shControl), the cells were transiently transfected with indicated cDNA and were used for cell proliferation assay (MTT) (D) or measuring the indicated metabolites (E).

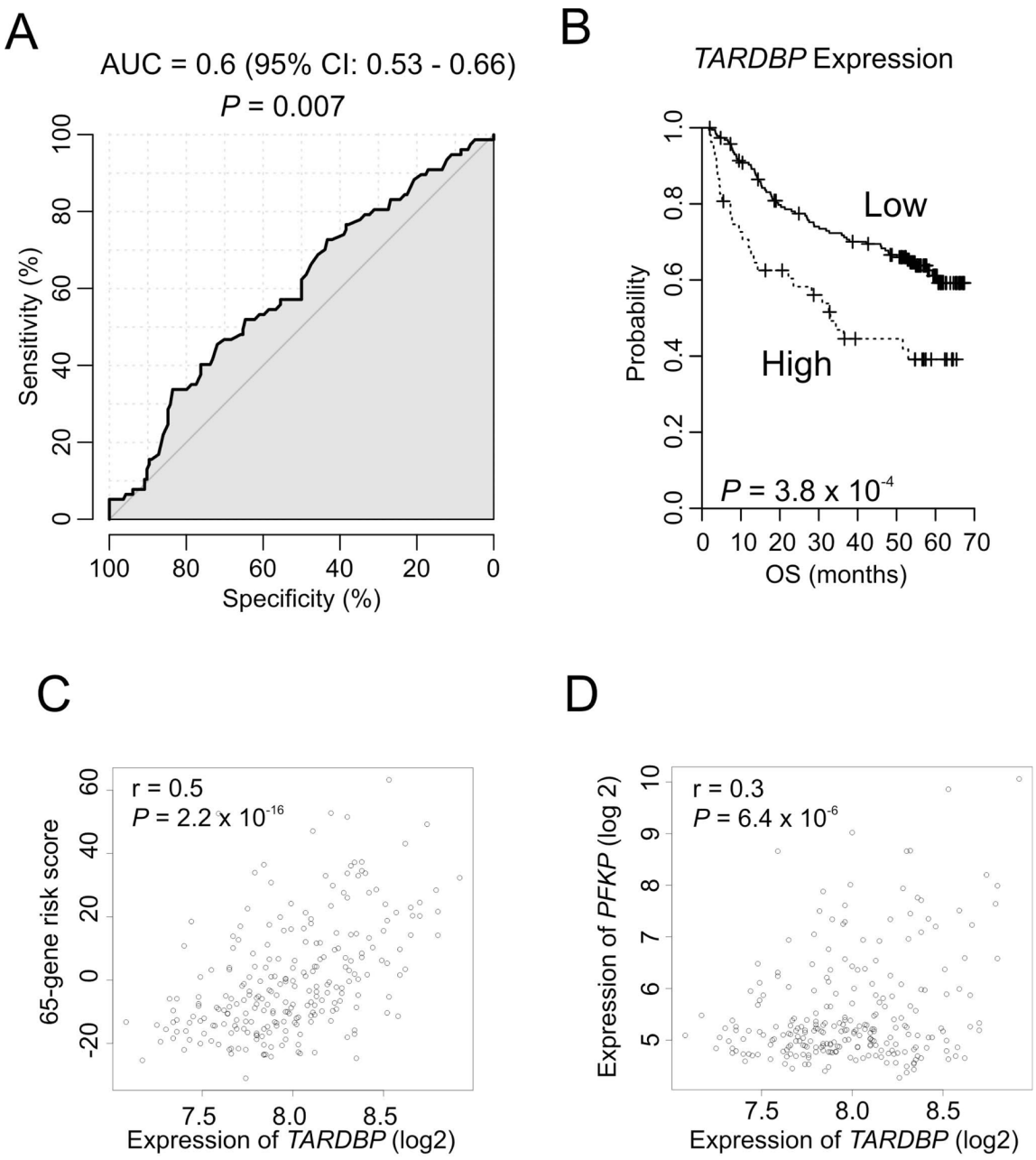

Fig. 6. Clinical relevance of TARDBP in patients with HCC

(A) Prognostic significance of TARDBP estimated by AUC from receiver operating characteristics analysis for 3-year OS. AUC: area under curve, CI: confident internal of AUC. (B) Kaplan–Meier plots of overall survival of HCC patients. Patients were stratified according to expression level of *TARDBP*. *P*-values were calculated with the log-rank test. (C) Scatter plots between 65-gene risk score and expression level of *TARDBP* in HCC (D) Scatter plots between expression level of *TARDBP* and *PFKP* in HCC.

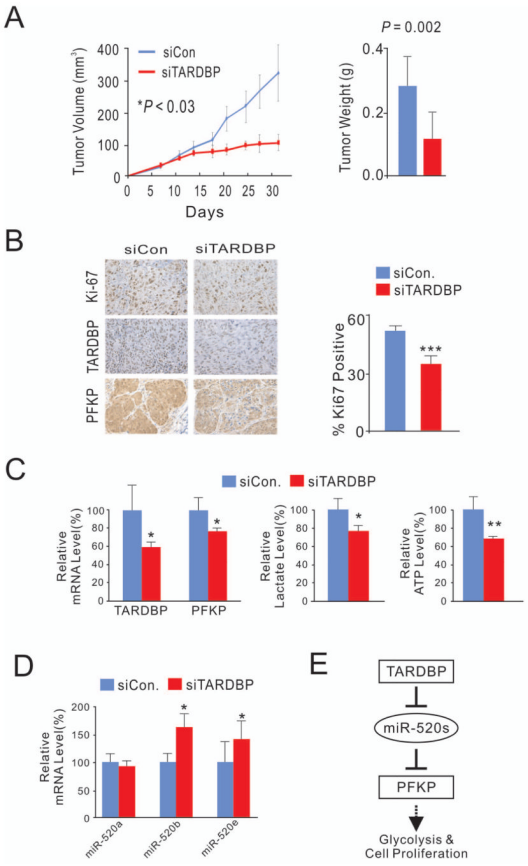

Fig. 7. Therapeutic potential of TARDBP

(A) Growth of SK-Hep1 tumors in mice treated with TARDBP siRNA-DOPC or control siRNA-DOPC. Tumor volume and weight were measured at day 33. (B) Immunostaining of Ki-67, TARDBP and PFKP in SK-Hep1 tumor tissues treated with TARDBP siRNA or control siRNA. (C) qRT-PCR assay with RNA and metabolites measured from SK-Hep1 tumor tissues treated with TARDBP siRNA or control siRNA. (D) qRT-PCR assay with miRNAs from SK-Hep1 tumor tissues treated with TARDBP siRNA or control siRNA. Data represent mean ± s.d. from three independent replicates (*P < 0.05, **P < 0.01, ***P < 0.005 by Student's t-test). (E) Schematic diagram of the regulatory pathway from TARDBP to glycolysis.