

TIPE drives a cancer stem-like phenotype by promoting glycolysis via PKM2/HIF-1 α axis in melanoma

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Abstract

TIPE (TNFAIP8) has been identified as an oncogene and participates in tumor biology. However, how its role in the metabolism of tumor cells during melanoma development remains unclear. Here, we demonstrated that TIPE promoted glycolysis by interacting with pyruvate kinase M2 (PKM2) in melanoma. We found that TIPE induced PKM2 dimerization, thereby facilitating its translocation from the cytoplasm to the nucleus. TIPE-mediated PKM2 dimerization consequently promoted HIF-1 α activation and glycolysis, which contributed to melanoma progression and increased its stemness features. Notably, TIPE specifically phosphorylated PKM2 at Ser 37 in an ERK-dependent manner. Consistently, the expression of TIPE was positively correlated with the levels of PKM2 Ser37 phosphorylation and cancer stem cell markers in melanoma tissues from clinical samples and tumor bearing mice. In summary, our findings indicate that the TIPE/PKM2/HIF-1 α signaling pathway plays a pivotal role in promoting cancer stem cell properties by facilitating the glycolysis, which would provide a promising therapeutic target for melanoma intervention.

eLife assessment

This **important** study investigates the molecular mechanisms underpinning how the tumor necrosis factor alpha-induced protein, TIPE, regulates aerobic glycolysis to promote tumor growth in melanoma. **Solid** data using multiple independent approaches provide new insights into the molecular mechanisms underpinning aerobic glycolysis, also known as the Warburg Effect, in melanoma cells. However, further investigation of a potential oncogenic effect of TIPE in melanoma patients is warranted and more advanced metabolomic and bioenergetic assays could be employed. The work will be of interest to biomedical researchers working in cancer and metabolism.

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Introduction

Melanoma is derived from the aberrant growth of melanocytes and is classified into several subgroups such as cutaneous melanoma (CM), uveal melanoma (UM), and mucosal melanoma (MM), based on its tissue locations (Han et al., 2021 [\[1\]](#)). Cutaneous melanoma (CM) is the major subgroup of melanoma. Accumulating evidence has revealed that metabolic reprogramming contributes to the tumorigenesis and progression of melanoma. Owing to its low incidence rate, targeting metabolism might be a promising therapeutic approach for the treatment of melanoma and offers a deeper understanding of its pathobiology (Brunner and Finley, 2022 [\[2\]](#); Faubert et al., 2020 [\[3\]](#)). However, melanoma-altered metabolic reprogramming remains poorly understood.

The Warburg effect, also known as aerobic glycolysis, is a part of metabolic reprogramming. It partly contributes to increased lactate levels regardless of oxygen availability in tumor cells, providing cancer cell tumorigenicity, progression, and chemoresistance (Guo et al., 2022 [\[4\]](#)). Pyruvate kinase isoform M2 (PKM2) is the key enzyme that irreversibly converts phosphoenolpyruvate (PEP) to pyruvate in the last step of glycolysis (Li et al., 2016 [\[5\]](#); Liu et al., 2021 [\[6\]](#)). It is shifted between catalytically inactive dimeric and active tetrameric forms, and the PKM2 dimer predominantly serves as a key glycolytic enzyme that provides advantages for tumor progression due to the Warburg effect (Liu et al., 2017 [\[7\]](#)). Post-translational modifications of PKM2, such as FGFR1-mediated Tyr105 phosphorylation and ERK1/2-dependent Ser37 phosphorylation, can promote PKM2 from a tetrameric to dimeric form, accelerating its translocation into the nucleus (Christofk et al., 2008 [\[8\]](#); Li et al., 2016 [\[5\]](#)). In the nucleus, PKM2 acts as a protein kinase that phosphorylates STAT3 (Chen et al., 2022a [\[9\]](#); Dhanesha et al., 2022 [\[10\]](#)) and Histone H3 (Niu et al., 2020 [\[11\]](#)). Interestingly, nuclear PKM2 also functions as a coactivator for several transcription factors, such as HIF-1 α (Luo et al., 2011 [\[12\]](#); Wang et al., 2014 [\[13\]](#)) or Oct4 (Yang et al., 2018 [\[14\]](#)), by which regulates metabolic gene transcription and promotes tumorigenesis or sustains cancer stem cell populations. Furthermore, HIF-1 α is constitutively activated in melanoma under both normal and hypoxic conditions and is accompanied by increased glycolysis (D'Aguanno et al., 2021 [\[15\]](#); Malekan et al., 2021 [\[16\]](#)). Therefore, the precise mechanisms underlying PKM2 activity regulation and its function as an HIF-1 α coactivator might provide a molecular basis for the melanoma targeted therapy.

The tumor necrosis factor alpha-induced protein 8 (TNFAIP8, TIPE) family is composed of four members: TIPE, TIPE1, TIPE2, and TIPE3 (Yang et al., 2022 [\[17\]](#)). Accumulating evidence suggests that TIPE is strongly associated with the development of various cancers by affecting cell proliferation, apoptosis, invasion, and metastasis (Goldsmith et al., 2017 [\[18\]](#); Nitire et al., 2018 [\[19\]](#)). Therefore, TIPE is broadly considered pro-cancerous (Padmavathi et al., 2018 [\[20\]](#)). However, we previously revealed that TIPE functions as a tumor suppressor in colitis and colitis-associated colon cancer (Lou et al., 2022 [\[21\]](#)), indicating that TIPE might serve a multifarious function in malignancies. Hitherto, the role of TIPE in melanoma remains unclear. In this study, we revealed that TIPE-mediated PKM2 Ser37 phosphorylation accelerates PKM2 translocation from the cytoplasm to the nucleus, thereby elevating its binding ability to HIF-1 α and hypoxia response elements (HREs), thus promoting metabolic reprogramming. TIPE-mediated metabolic reprogramming can confer tumorigenesis and stem-like phenomena to melanoma cells. This process represents an important regulator of the TIPE family for regulating aerobic glycolysis, paving the way for cancer-specific metabolism in response to low-oxygen challenge.

Results

TIPE increases aerobic glycolysis in melanoma cells

To characterize how TIPE performs biological functions in melanoma, we examined its expression levels in melanoma cell lines. The results showed that A375 and A875 cells had relatively higher TIPE expression than G361 and MM96L cells. Therefore, we downregulated TIPE expression in A375 cells and overexpressed TIPE in G361 cells to perform further experiments (Fig.S1). Furthermore, we demonstrated that TIPE promotes melanoma tumorigenesis both *in vitro* and *in vivo* (Fig.S2). Consequently, we performed RNA sequencing analysis by overexpressing TIPE in G361 cells (Fig. 1A) to examine the underlying mechanisms involved in the progression of melanoma. The results revealed that the overexpression of TIPE dramatically promoted expression of genes regarding glycolysis and the HIF-1 α pathway (Fig. 1B, C). Since HIF-1 α pathway participates in metabolic reprogramming, especially aerobic glycolysis as well (Baik et al., 2019), we assumed that TIPE promotes melanoma progression might via the regulation of metabolic reprogramming. Therefore, we performed a metabolomic analysis after interfering with TIPE in A375 cells. Interestingly, the results indicated that the aerobic glycolysis pathway, including pyruvate and lactate, was significantly inhibited in melanoma cells after TIPE silencing (Fig.S3, Fig. 1D), confirming that TIPE facilitates glycolytic metabolism in melanoma. Consequently, ATPase activity, lactate secretion, and ATP content assays were performed to further confirm TIPE-induced glycolytic changes. The results demonstrated that TIPE decreased ATPase activity and ATP content while promoting lactate production (Fig. 1E-G). Additionally, we measured the extracellular acidification rate (ECAR) after the overexpression or downregulation of TIPE using a Seahorse Bioscience Flux Analyzer. The results also revealed that TIPE promoted glycolysis and glycolytic capacity in melanoma cells (Fig. 1H-K).

Furthermore, we investigated whether TIPE affected HIF-1 α transcriptional activity or its expression levels. The results demonstrated that TIPE increased the expression of HIF-1 α at both mRNA and protein levels (Fig. S4). Interestingly, TIPE can dramatically activate the hypoxia response element(HRE) reporter activity, with which HIF-1 α is more prone to recognize the specific DNA motif to regulate metabolic gene transcription(Chen et al., 2022b)(Fig. 1L).

Collectively, above results demonstrate that TIPE plays a crucial role in promoting melanoma glycolysis. However, the impact of TIPE on the Warburg effect by regulating HIF-1 α activity requires further investigation.

TIPE interacts with PKM2, promotes its dimerization and nuclear import

To further study the functions of TIPE in melanoma, we used the Co-IP/MS method to bait the potential binding partners of TIPE (the candidate interacting proteins of TIPE are listed in Table S1). Interestingly, we found that PKM2, the key enzyme in aerobic glycolysis, might be a new partner of TIPE in melanoma (Fig. 2A) (Fig. S5). Next, we performed Co-IP assays to confirm the interactions between TIPE and PKM2. The results revealed that exogenously overexpressed Flag-tagged PKM2 interacted with exogenously overexpressed HA-tagged TIPE (Fig. 2B, C). Furthermore, endogenous interactions between PKM2 and TIPE were confirmed in the A375 cells (Fig. 2D, E). GST-pull down assays demonstrated that TIPE directly bound to PKM2 *in vitro* (Fig. 2F). Moreover, we performed Doulink assay to further demonstrate their endogenous interactions. The results also showed that endogenous interactions between TIPE and PKM2 were confirmed in G361 cells (Fig. 2G). Based on these interactions, we identified the binding regions between TIPE and PKM2. We generated four fragments of PKM2, including amino acids 1-390(NA1BA2), 45-390(A1BA2), 1-219(NA1B) and 220-390(A2), based on their structural domains

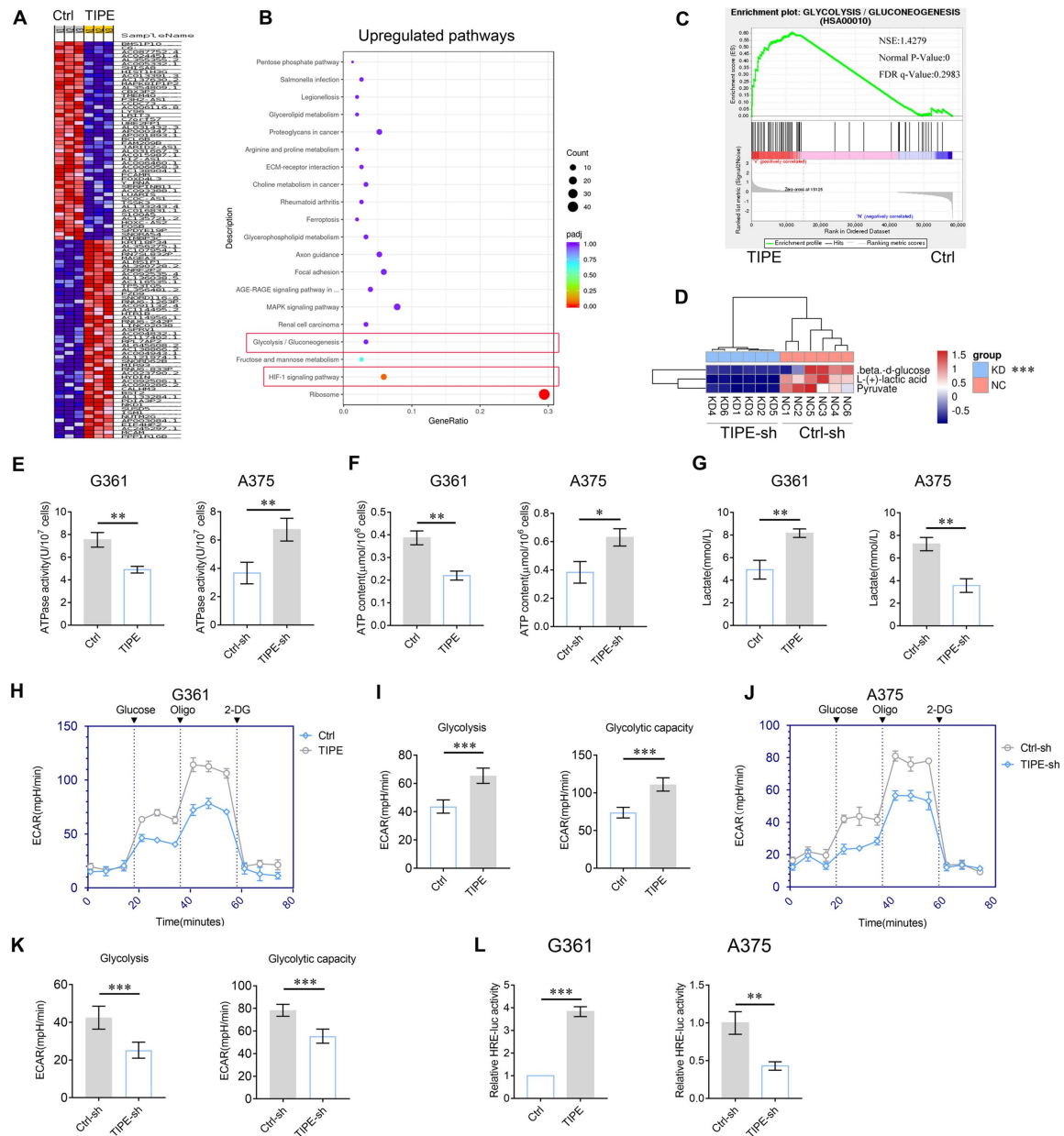


Figure 1.

Effect of TIPE on melanoma cell glycolysis. A, B.

Transcriptomics analysis by unsupervised hierarchical clustering and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that TIPE increased glycolysis and HIF-1 α pathways. **C.** GSEA analysis of glycolysis showed that TIPE enhanced glycolysis compared to the control. **D.** Untargeted metabolomics analysis indicated that interfering with TIPE decreased the glycolysis pathway. **E-G.** TIPE decreases ATPase activity and ATP content and increases lactate levels. **H, I.** Overexpression of TIPE promotes glycolysis and glycolytic capacity according to ECAR analysis. **J, K.** Interfering with TIPE decreased glycolysis and glycolytic capacity using ECAR analysis. **L.** TIPE significantly activated HRE activity. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The data represent the means $\pm$ SEM of 3 replicates.

(Fig. 2H). The results showed that except for the A2 domain, the NA1BA2, A1BA2, and NA1B domains of PKM2 were necessary for its interaction with TIPE (Fig. 2I). Finally, the TIPE protein was divided into two fragments, including amino acids 1-100 (M1) and 101-198 (M2). The data showed that the amino acids 1-100(M1) and 101-198(M2) of TIPE interacted with PKM2 (Fig. 2J). Due to both domains of TIPE can interact with PKM2, we believe that the A2 domain of PKM2 might have important structural effects that could affect interactions with TIPE. Taken together, these data revealed that TIPE interacts with PKM2.

Since TIPE interacts with PKM2 in melanoma, we determined whether TIPE increases aerobic glycolysis by affecting PKM2 expression or its activity. The results showed that TIPE did not influence the expression of PKM2 at either the mRNA or the protein level (Fig. S6). Intriguingly, we demonstrated that dimeric PKM2 was reduced in TIPE-silenced A375 cells (Fig. 2K) and was increased following TIPE overexpression in G361 cells (Fig. 2L). Normally, a dimeric form of PKM2 with low affinity for its substrate, phosphoenolpyruvate (PEP), and a tetrameric form have a high PEP affinity. Moreover, the dimeric form of PKM2 is more prone to translocation into the nucleus and thus exerts protein kinase activity, which confers the Warburg effect and tumorigenesis (Zhou et al., 2022). Western blot analysis of nuclear and cytosolic fractions from A375 cells revealed that PKM2 primarily resided in the cytoplasm in which TIPE was downregulated. Likewise, the nuclear level of PKM2 was increased after overexpression of TIPE in G361 cells (Fig. 2M). Furthermore, we examined the distribution of PKM2 after TIPE overexpression by using a Thermo Fisher EVOS microscopic analysis. As shown in Fig. 2N, PKM2 was predominantly present in the nucleus after TIPE overexpression, and this phenomenon was diminished by the administration of a PKM2 dimer formation suppressor (TEPP-46, 100 μ M). It has been shown that dimeric PKM2 promotes tumor progression by regulating the Warburg effect (Zhou et al., 2022). Thus, we speculated that TIPE restores the nuclear levels of PKM2 to promote melanoma tumorigenicity via a dimeric PKM2-dependent Warburg effect.

TIPE requires PKM2 for transcriptional activation of HIF-1 α

Accumulating evidence has revealed that tetrameric PKM2 is enzymatically active towards pyruvate and acts as a transcriptional coactivator, while it dissociates into a dimer (Wei et al., 2020). Consequently, the dimeric form of PKM2 becomes a coactivator of HIF-1 α to transcriptionally activate glycolytic genes in favor of the Warburg effect (Fig. 3A) (Ouyang et al., 2018). As PKM2 and HIF-1 α both play important roles in the metabolic reprogramming process, it is reasonable to believe that the interactions between TIPE and these two molecules could affect the glycolysis in melanoma. Thus, we investigated whether TIPE promoted the transcriptional activation of HIF-1 α in a PKM2-dependent manner.

To confirm the effect of TIPE activation on HIF-1 α transcriptional activity, we tested the ability of TIPE to activate the HRE reporter activity in HRE-luciferase plasmid transfected HEK-293T cells. Not surprisingly, significant augmentation of HRE promoter activation by TIPE was observed, especially when combined with PKM2 (Fig. 3B). Consequently, TIPE activated HRE reporter activity in a dose-dependent manner, and this phenomenon was reversed after PKM2 knockdown, demonstrating that TIPE has critical effects on the activation of HIF-1 α transcription in a PKM2-dependent manner (Fig. 3C). HIF-1 α targeted genes such as *LDHA* and *GLUT1* were also upregulated by TIPE in a dose-dependent manner and were reversed by interfering with PKM2 (Fig. 3D, E). Due to PKM2 cooperated with HIF-1 α to activate glycolytic genes during the Warburg effect, we used the Duolink method to confirm whether TIPE affects the endogenous interaction between HIF-1 α and PKM2. The results showed that the interaction between HIF-1 α and PKM2 was decreased by TIPE interference, whereas the interaction was increased when TIPE was overexpressed (Fig. 3F, G). More interestingly, TIPE enhanced the PKM2/HIF-1 α interaction in the nucleus, further indicating that TIPE induced PKM2 nuclear translocation and thus promoted its interaction with HIF-1 α (Fig. 3H). In addition, the Co-IP assay revealed that TIPE elevated this interaction in a dose-dependent manner (Fig. 3I).

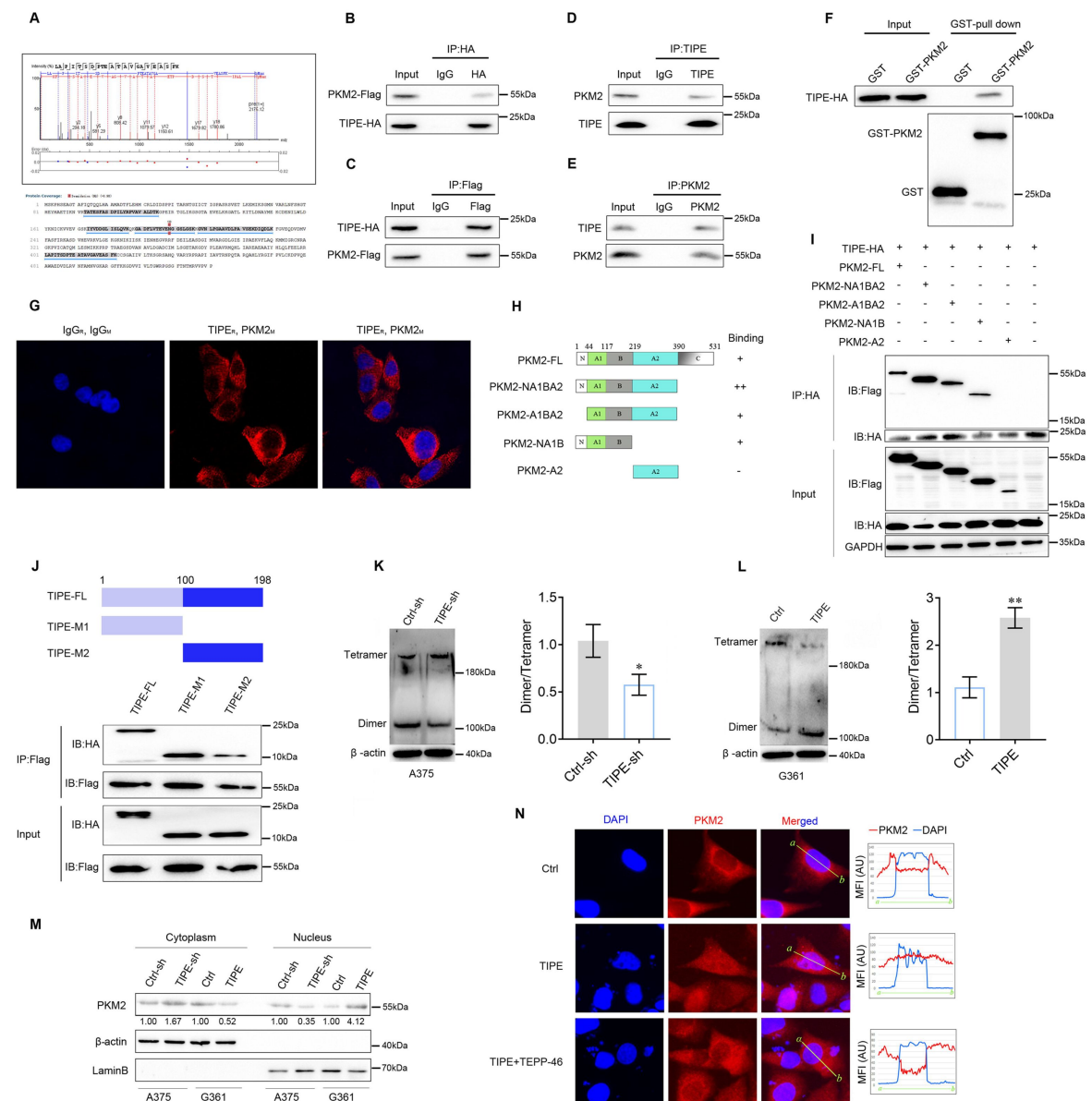


Figure 2.

TIPE interacts with PKM2 to govern its nuclear import in a dimeric form dependent manner. **A.**

Co-IP/MS analysis demonstrated that PKM2 interacted with TIPE in A375 cells. The results indicated that there were 5 peptides of PKM2 were detected to interact with TIPE (below), and one of which was shown at the upper. **B, C.** Immunoprecipitation (IP) and western blot analysis of the exogenous TIPE/PKM2 proteins interaction in the HEK-293T cells co-transfected with Flag-tagged PKM2 and HA-tagged TIPE. **D, E.** IP and western blot analysis of the endogenous TIPE/PKM2 proteins interaction in the A375 cells. **F.** GST-pull down assay analysis of TIPE/PKM2 proteins interaction using purified GST-tagged PKM2 and TIPE-HA. **G.** TIPE (rabbit source, TIPE_R) endogenously interacted with PKM2(mouse source, PKM2_M) in G361 cells by using the Doulink assay. The results suggested that TIPE interacted with PKM2 to form a red color complex. The red color signal is generated only when the two proteins are too close to interaction. **H, I.** IP and western blot analysis of HA-tagged TIPE and Flag-tagged PKM2 fragment protein interaction in HEK-293T cells. **J.** IP and western blot analysis of the Flag-tagged PKM2 and HA-tagged TIPE fragment protein interaction in HEK-293T cells. **K.** Interfering TIPE decreased PKM2 dimeric formation but increased tetramer formation, as analyzed by BN-PAGE with β -actin as a loading control. **L.** Overexpression of TIPE increased PKM2 dimeric formation and decreased tetramer formation. **M.** Western blot showed that TIPE promoted PKM2 translocation into the nucleus. **N.** TIPE enhanced PKM2 translocation into the nucleus, and this phenomenon was diminished by the administration of TEPP-46 (100 μ M). * P <0.05; ** P <0.01. The data represent the means $\pm$ SEM of 3 replicates.

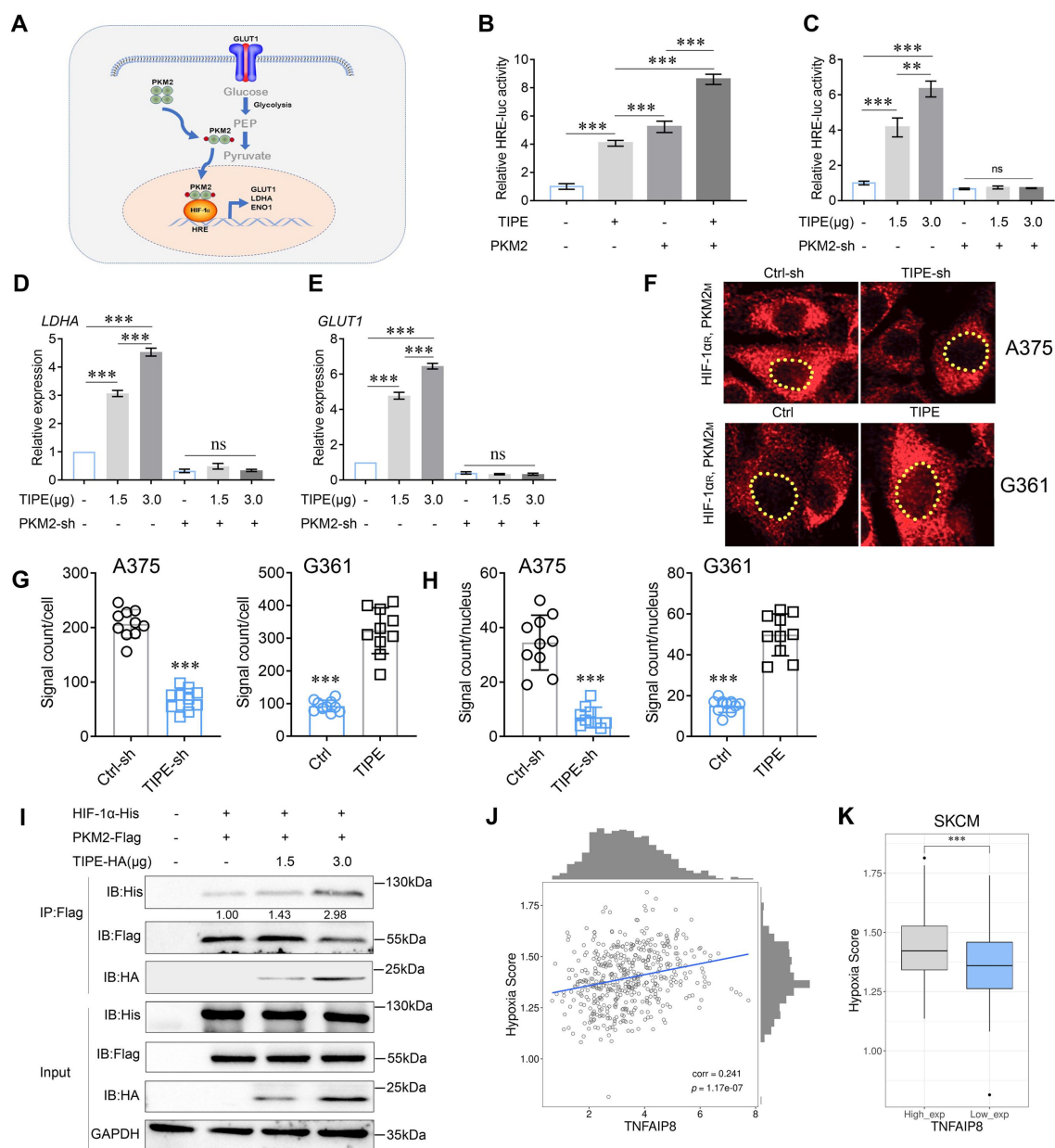


Figure 3.

TIPE promotes HIF-1 α transcription in a PKM2-dependent manner. **A.** Proposed molecular mechanism by which dimeric PKM2 regulates cell proliferation and glycolysis by modulating HIF-1 α activity. **B.** TIPE, especially when combined with PKM2, boosts relative HRE luciferase activity, as examined by luciferase reporter assay. **C.** TIPE promoted HRE activity in a dose- and PKM2-dependent manner. **D, E.** TIPE increases HIF-1 α targeted genes, including *LDHA* and *GLUT1*, in a dose- and PKM2-dependent manner. **F, G.** TIPE promoted endogenous interaction between PKM2 and HIF-1 α in melanoma cells using a Doulink assay. Interference of TIPE in A375 cells promoted the interaction between PKM2 and HIF-1 α (upper) compared to that overexpression of TIPE in G361 cells decreased their interaction (lower). The density of the red color signaling means the interactive strength between PKM2 and HIF-1 α affected by TIPE. **H.** TIPE enhanced the PKM2/HIF1 α interaction in the nucleus. **I.** TIPE increased the exogenous interaction between PKM2 and HIF-1 α in a dose- dependent manner in HEK-293T cells. **J.** TCGA dataset revealed that TIPE has a positive relationship with hypoxia score in melanoma. **K.** Higher expression of TIPE is associated with a relatively higher hypoxia score in melanoma. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The data represent the means $\pm$ SEM of 3 replicates.

Moreover, we analyzed the correlation between TIPE and hypoxia scores using TCGA dataset. Interestingly, we found that the hypoxia score was significantly related to the expression of TIPE (**Fig.3J**) (**Table S2**), and the TIPE higher expression group had a higher hypoxia score than the lower expression group in melanoma (**Fig.3K**).

Collectively, these results demonstrated that TIPE has critical effects on the transcriptional activation of HIF-1 α in a PKM2-dependent manner.

TIPE promotes HIF-1 α transcriptional activation via PKM2 Ser37 phosphorylation

The presence of PKM2 in the nucleus enhances glucose metabolism by regulating HIF-1 α (Luo et al., 2011). Our results preliminarily demonstrate that TIPE promotes PKM2 dimerization and nuclear import, thereby increasing HIF-1 α transcriptional activation and promoting aerobic glycolysis. Normally, Erk1/2-dependent phosphorylation of PKM2 at serine 37 (Ser37) and FGFR1-dependent phosphorylation of PKM2 at tyrosine 105 (Tyr105) (Li et al., 2016; Zhou et al., 2018) are indicative of its dimerization and translocation, further promoting the Warburg effect and serving as a transcription factor coactivator (Novoyatleva et al., 2019; Wei et al., 2020). As shown in **Fig.4A**, interference with TIPE in A375 cells blocked PKM2 Ser37 phosphorylation, and overexpression of TIPE in G361 cells caused Ser37 phosphorylation compared to controls. However, the phosphorylation of PKM2 Tyr105 did not show any obvious changes. Consequently, we hypothesized that this transformation caused by Ser37 phosphorylation affects the interaction between TIPE and PKM2. As expected, the mutation of PKM2 at S37A blocked its interaction with TIPE, but not with the Y105D mutation (**Fig.4B**). Interestingly, this mutation also interfered with the interaction between PKM2 and HIF-1 α , which was elevated by TIPE overexpression (**Fig.4C**). A previous study has revealed that TIPE promotes chemoresistance in acute myeloid leukemia (AML) by activating the ERK signaling pathway (Pang et al., 2020). In addition, phosphorylation of PKM2 at serine 37 is ERK1/2-dependent manner (Yang et al., 2012). TIPE does not serve as a phosphorylase to perform such post-translational modifications directly. Taken together, these results indicate that TIPE promotes PKM2 dimerization depending on ERK signaling. As expected, the ERK inhibitor, U0126, inhibited PKM2 Ser37 phosphorylation induced by TIPE overexpression (**Fig.4D**). To investigate whether TIPE affects the binding affinity of PKM2 to the Hif-1 α specific binding site of the *LDHA* and *GLUT1* promoter using chromatin immunoprecipitation qPCR (ChIP-qPCR) analysis, we found that TIPE increased the binding of PKM2 to the *LDHA* and *GLUT1* promoters. This phenomenon, caused by the overexpression of TIPE, was diminished after the administration of TEPP-46, indicating that TIPE promoted the binding of PKM2 to the *LDHA* and *GLUT1* promoters in a dimeric PKM2-dependent manner (**Fig.4E**, **F**). Finally, we demonstrated that the augmentation of TIPE on HRE promoter activation or HIF-1 α targeted genes such as *LDHA* and *GLUT1* was blocked by the S37A mutation (**Fig.4G-I**).

In brief, we revealed that TIPE promoted HIF-1 α transcriptional activation via ERK-dependent PKM2 Ser37 phosphorylation.

TIPE facilitates melanoma tumorigenesis and aerobic glycolysis in a dimeric PKM2-dependent manner

Several studies have demonstrated that dimeric PKM2 increases tumor progression by regulating the Warburg effect. Therefore, we speculated that TIPE promotes melanoma growth through a dimeric PKM2-dependent Warburg effect. To demonstrate this, we knocked down TIPE in A375 cells and found that cell proliferation was decreased. Interestingly, we found that Pyridoxine, a PKM2 dimer formation inducer (Wei et al., 2020), was sufficient to abrogate the TIPE silencing-mediated cell proliferation (**Fig.5A**). Consistently, TEPP-46, a PKM2 dimer formation suppressor, was sufficient to reverse the increased proliferative capacity induced by TIPE-overexpression in G361 cells (**Fig.5B**). Clone formation ability (**Fig.5C**, **D**) and *in vivo* experiments (**Fig.5E-G**)

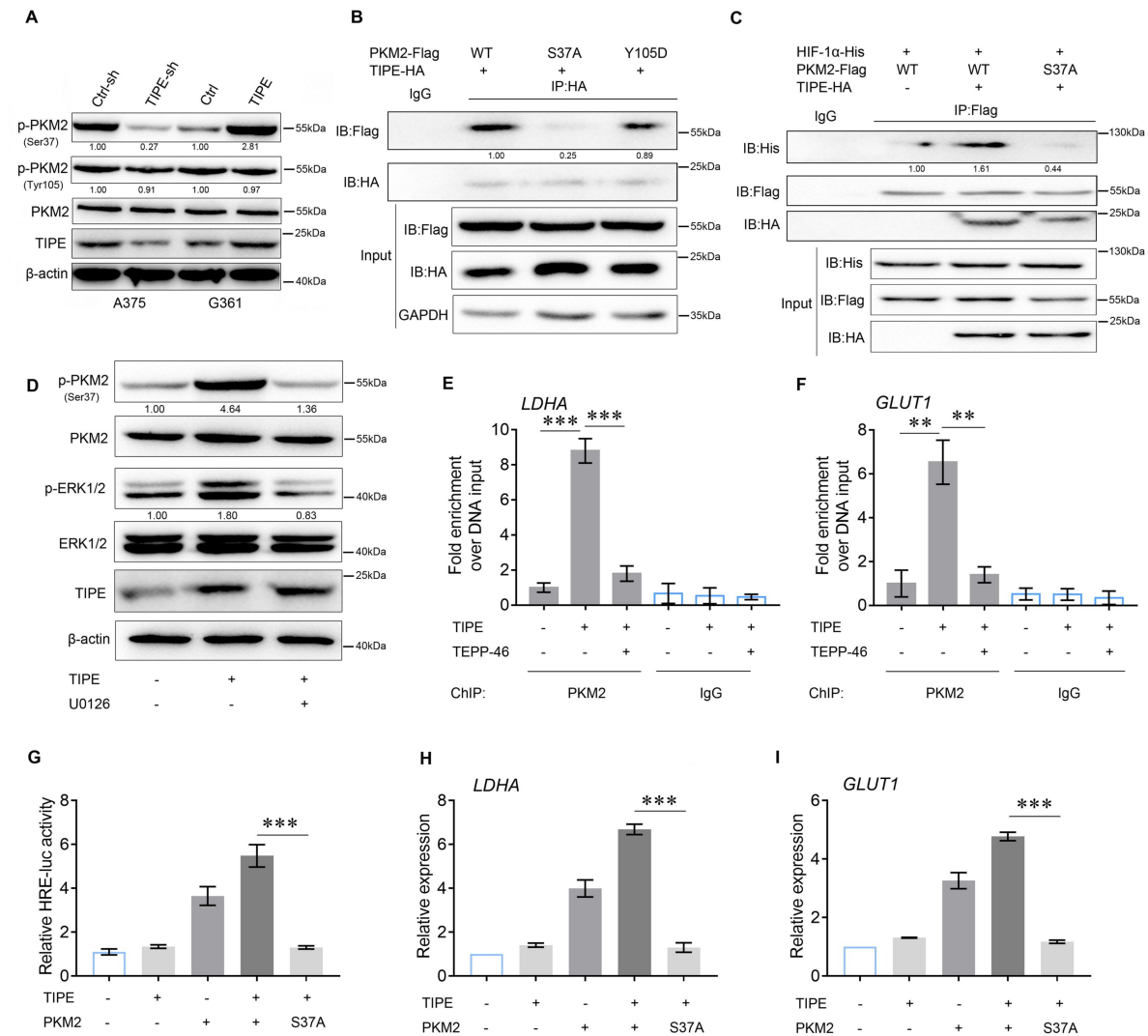


Figure 4.

TIPE increases HIF-1α activity depending on PKM2 Ser37 phosphorylation. **A.** TIPE enhanced PKM2 Ser37 phosphorylation, but not that of Tyr105. **B.** PKM2 Ser37, but not Tyr105, increased its interaction with TIPE. **C.** PKM2 Ser37 mutation(S37A) hampered its interaction with HIF-1α promoted by TIPE. **D.** TIPE elevated PKM2 Ser37 phosphorylation in an ERK-dependent manner. **E, F.** TIPE enhanced PKM2 binding to the HRE for *LDHA* and *GLUT1* in a dimeric form dependent manner. **G.** PKM2 Ser37 mutation(S37A) inhibited the HRE activity induced by TIPE. **H, I.** PKM2 Ser37 mutation(S37A) decreased the expression of *LDHA* and *GLUT1* that promoted by TIPE. **P<0.01; ***P<0.001. The data represent the means ± SEM of 3 replicates.

also demonstrated this phenomenon. Interestingly, we found that decreased ECAR, glycolysis, and glycolytic capacity induced by TIPE interference could be fully antagonized by Pyridoxine in A375 cells (**Fig.5H** [↗](#)-**J**). In addition, TEPP-46 reversed the ECAR, glycolysis, and glycolytic capacity induced by TIPE overexpression in G361 cells (**Fig.5K** [↗](#)-**M**).

Lastly, to further demonstrate how TIPE affects the PKM2/HIF-1 α pathway during the Warburg effect, we revealed that inhibition of PKM2 dimerization can rescue TIPE-induced cell proliferation. However, this phenomenon was diminished by the activation of HIF-1 α , demonstrating that TIPE promotes melanoma cell proliferation via dimeric PKM2 dependent HIF-1 α activation (**Fig.5N** [↗](#), **O**). More interestingly, we evidenced that suppression of PKM2 dimerization inhibited the TIPE- mediated Warburg effect. In addition, this phenomenon was diminished by the activation of HIF-1 α (**Fig.5P** [↗](#), **Q**).

Taken together, these data suggest that TIPE regulates cell growth and the Warburg effect by inducing the formation of dimeric PKM2, and thus activating the HIF-1 α pathway.

TIPE accelerates melanoma cells stem-like phenomenon via promoting PKM2 dimerization under normoxic conditions

Increasing evidence has shown that the HIF-1 α pathway is associated with acquisition of cancer stem-like properties(Weinstein et al., 2022 [↗](#); Zhu et al., 2022 [↗](#)). To confirm the effects of TIPE on the stemness of melanoma cells under normoxic conditions, we examined the stemness-associated features *in vitro*. First, we measured cancer stem-like phenotype markers, including *NANOG*, *NOTCH*, *OCT3/4*, *SOX2* and *BMI-1*. The results showed that the markers were increased after overexpression of TIPE in G361 cells and were reduced after TIPE interference in A375 cells by using *qPCR* method. More interestingly, neural crest stem cell markers (including *NES* and *SOX10*) that are more relevant to melanoma biology were also greatly changed (**Fig.6A** [↗](#), **B**). We further revealed that TIPE increased the migration ability and increased sphere formation (**Fig.6C** [↗](#), **D**). In addition, TIPE significantly increased the chemosensitivity of melanoma cell to sorafenib (**Fig.6E** [↗](#), **F**). Next, we examined the tumorigenicity of melanoma cells with TIPE knockdown *in vivo* by injecting Ctrl-sh or TIPE-sh A375 cells subcutaneously into nude mice at three dilutions (1×10^6 , 1×10^5 and 1×10^4) and allowing them to grow for four weeks. The Confidence intervals (CIs) for 1/ (stem cell frequency) using extreme limiting dilution were calculated as previous reported(Hu and Smyth, 2009 [↗](#); Niu et al., 2021 [↗](#)). The estimated CI for the frequency of CSCs in the TIPE knockdown group was 202749, compared with 18441 in the control group ($P < 0.001$) (**Fig.6G** [↗](#)) (**Table S3**, 4). Moreover, CD44⁺ subpopulations were significantly elevated, as detected using a fluorescence-activated cell sorting (FACS) assay (**Fig.6H** [↗](#), **I**).

Because TIPE promotes HIF-1 α transcriptional activation in a dimeric PKM2-dependent manner, we performed a sphere formation assay by overexpressing TIPE in G361 cells. The results showed that TEPP-46 partially reversed tumorsphere formation induced by TIPE overexpression. Moreover, overexpression of HIF-1 α diminished this phenomenon, indicating that TIPE-induced sphere formation depended on dimeric PKM2-dependent HIF-1 α activation (**Fig.6J** [↗](#)). In addition, the increased CD44⁺ subpopulations of G361 cells induced by TIPE were decreased by the administration of TEPP-46, and could be rescued by overexpression of HIF-1 α (**Fig.6K** [↗](#)).

These findings strongly suggest that TIPE maintains melanoma cell stemness via dimeric PKM2-dependent HIF-1 α activation.

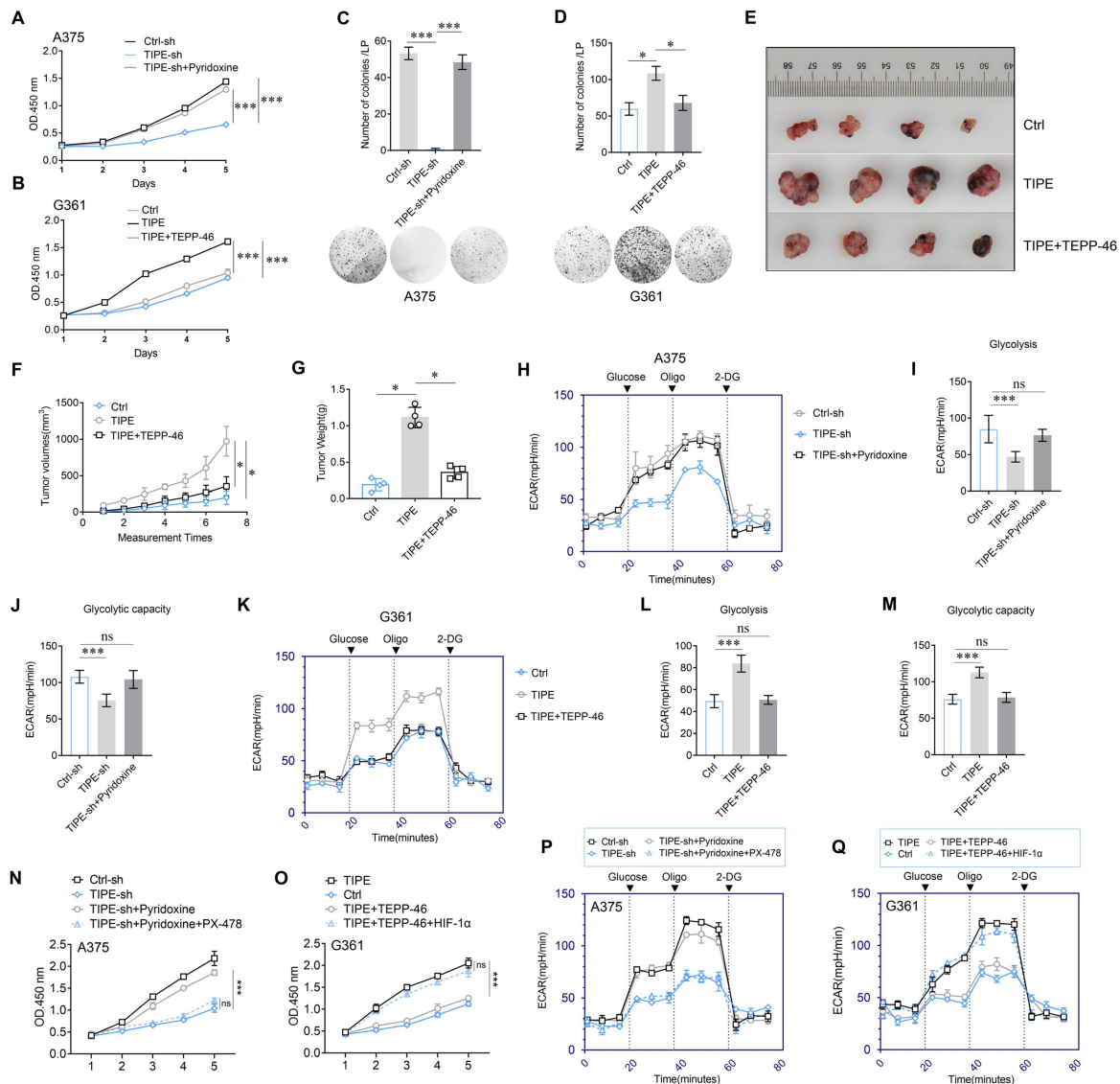


Figure 5.

TIPE promotes melanoma cells proliferation and glycolysis depending on PKM2 dimerization. A.

Pyridoxine (which facilitates PKM2 dimerization) treatment reversed the inhibition of cell proliferation that induced via interfering TIPE. **B.** Administration of TEPP-46 rescued TIPE overexpression induced cell proliferation. **C, D.** TIPE promoted melanoma clone formation in a dimeric PKM2-dependent manner. **E-G.** *In vivo* experiments showed that TIPE promoted melanoma proliferation via the dimerization of PKM2. **H-J.** Activation of PKM2 dimerization reversed the inhibition of glycolysis and glycolytic capacity after TIPE interfering. In contrast, inhibition of PKM2 dimerization altered this phenomenon (**K-M**). **N.** Pyridoxine increased the cell proliferation that was inhibited by TIPE-sh. It was rescued by inhibition of HIF-1α. **O.** TEPP-46 inhibited the TIPE-induced cell proliferation, and was rescued by overexpression of HIF-1α. **P.** Pyridoxine enhanced the Warburg effect that was suppressed by TIPE-sh, and it was diminished via administration of PX-478 (a HIF-1α inhibitor). **Q.** TEPP-46 can suppression of the Warburg effect that was increased by overexpression of TIPE, this phenomenon was diminished by overexpression of HIF-1α. * $P < 0.05$; *** $P < 0.001$. The data represent the means $\pm$ SEM of 3 replicates.

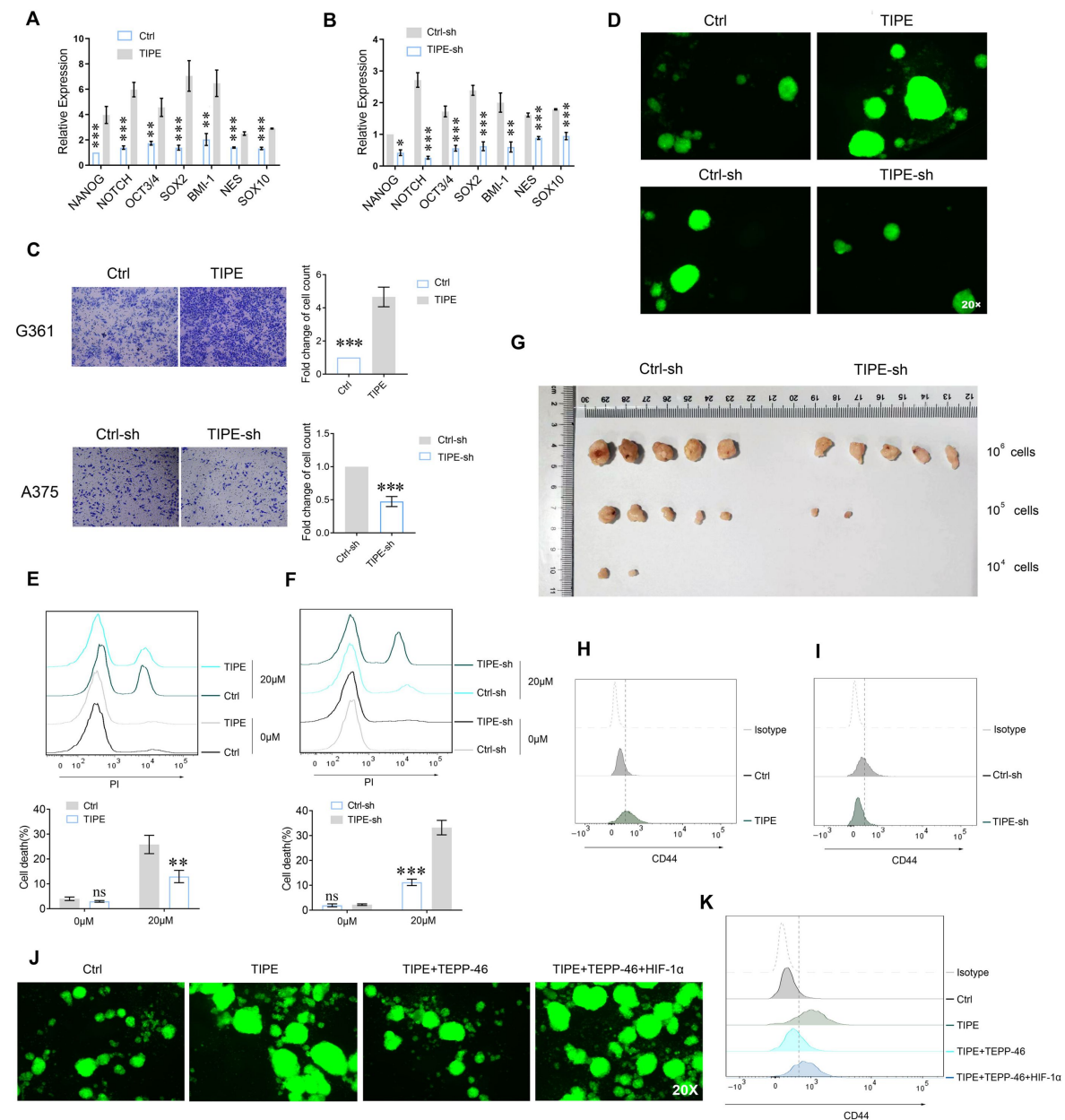


Figure 6.

TIPE fostered PKM2 dimerization increases melanoma stem-like phenomenon. A, B.

TIPE increased the cancer stem-like phenotype markers, including *NANOG*, *NOTCH*, *OCT3/4*, *SOX2*, *BMI-1*, *NES* and *SOX10*, measured by qPCR. **C-F**. The effects of TIPE on the stemness of melanoma cells are shown by *in vitro* cell migration (**C**), sphere formation (**D**), and chemoresistance (**E**, **F**). **G**. Limiting dilution xenograft formation of A375 cells with TIPE interference. Nude mice were subcutaneously injection of indicated cells. After about 60 days later, the mice were sacrificed and the confidence intervals (CIs) for 1 / (stem cell frequency) was calculated. **H**, **I**. TIPE increased the expression of CD44⁺ cells in melanoma. **J**, **K**. The effects of TIPE on the stemness of melanoma cells following the administration of TEPP-46, and this phenomenon was reversed by overexpression of HIF-1α, as evidenced by sphere formation (**J**) and CD44⁺ cell population (**K**). **P*<0.05; ***P*<0.01; ****P*<0.001. The data represent the means ± SEM of 3 replicates.

TIPE is positively correlated with the levels of PKM2 Ser37 phosphorylation and cancer stem cell markers

We analyzed the expression of TIPE in melanoma and benign skin tumors, and the results showed that the expression of TIPE was dramatically increased compared to controls using a tissue chip (Fig.7A, B). Furthermore, the expression of TIPE in primary group (non-lymph node metastasis) was lower than that of the metastatic group (lymph node metastasis) (Fig.7C). To determine if there was a correlation between TIPE and PKM2 Ser37 levels and other indicators such as Ki67 and hypoxia related gene (LDH), their expression were analyzed by IHC staining. The results showed that TIPE expression was positively correlated with PKM2 Ser37, Ki67 and LDH expression (Fig.7D). However, the relationship between TIPE levels and melanoma patient outcome was not analyzed due to lacking of the survival data in the tissue chip.

Consistent with the above findings, immunohistochemistry (IHC) results showed that TIPE, PKM2 Ser37, LDH, and CSCs marker CD44 were inhibited in A375 TIPE-interfering cell-derived xenografts (Fig.7E). Lastly, TCGA dataset revealed that TIPE has a positive relationship with CSCs markers including *BMI1*, *NANOG*, *NOTCH1*, and *OCT3/4(POU5F1)* in melanoma (Fig.7F-I).

These results further demonstrate that TIPE maintains cancer stem cell phenotypes in a PKM2 dimerization manner.

Discussion

Accumulating evidence has revealed that TIPE serves as an oncogene and a negative regulator of apoptosis. Its overexpression is positively associated with the development of various cancers, including liver, lung, and breast cancers (Lou et al., 2022; Niture et al., 2018). However, Chen et al. reported that TIPE initially inhibits cancer cell proliferation but promotes tumor migration and metastasis (Li et al., 2021). In addition, a previous report has demonstrated that TIPE functions as a tumor suppressor in certain inflammatory carcinogenesis (Lou et al., 2022). These results suggest that TIPE plays a variety of roles in cancer progression. Interestingly, Niture et al. reported that TIPE promotes prostate cancer progression by regulating oxidative phosphorylation and glycolysis (Niture et al., 2021). However, the mechanism through which TIPE regulates metabolic reprogramming remains unclear. Here, we demonstrate that TIPE facilitates the Warberg effect by promoting dimeric PKM2-mediated HIF-1 α transactivation in melanoma. Therefore, targeting TIPE may offer a novel therapeutic approach for melanoma treatment.

Mammalian pyruvate kinase consists of four isoforms, M1, M2, L, and R, which are encoded by two distinct genes (PKM and PKLR) (Valentini et al., 2002). Although these isoforms share similar features, only PKM2 exhibits the lowest basal enzymatic activity and is capable of configuration transformation between the tetramer and dimer, meeting differential metabolic demands (Morgan et al., 2013; Puckett et al., 2021). Cancer cells shift towards the preferential expression of the specific isoform of PKM2, especially its dimeric form, and serve as a pivotal metabolic enzyme that promotes the activation of oncogenic pathways in tumors (Icard et al., 2022). Normally, PKM2 enzymatic activity is accurately modulated in a variety of ways, such as post-translational modifications or metabolite binding, to maintain balanced levels in tumor cells (Puckett et al., 2021). Here, we showed that TIPE promoted melanoma tumorigenesis by promoting glycolysis. The results showed that TIPE interacts with PKM2 and may participate in glycolysis. However, TIPE did not influence the expression of PKM2 at either the mRNA or the protein level. Because of the modulation of PKM2 kinetic properties primarily via its dimer and tetramer configurations, we demonstrated that TIPE could promote its dimeric form transformation, increasing PKM2 residing in the nucleus. However, the downstream signaling pathways that are mediated by TIPE in melanoma remain largely unknown.

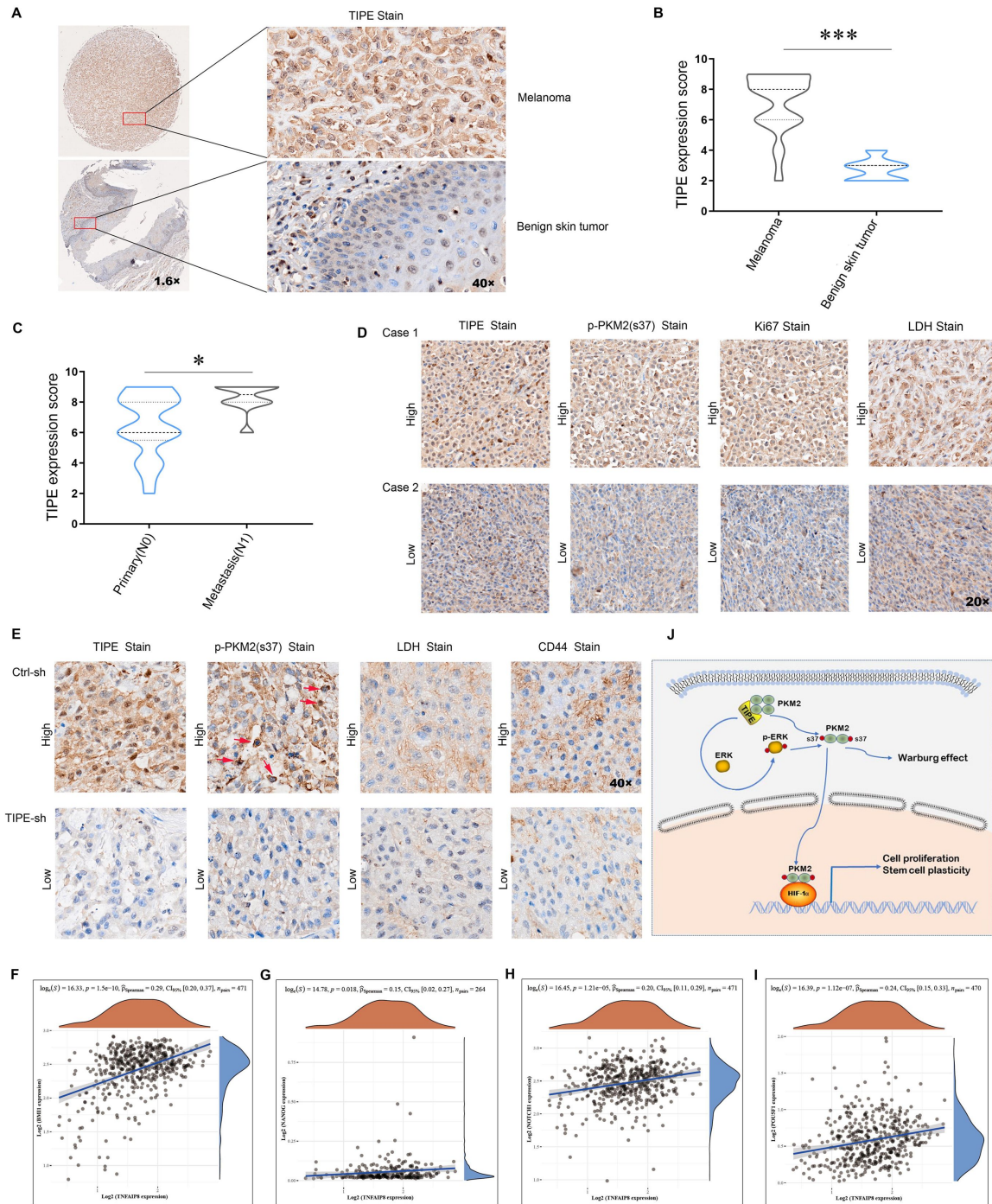


Figure 7.

TIPE positively correlated with cancer stem cell markers and the levels of p-PKM2(Ser37). A, B.

Higher expression of TIPE was observed in melanoma tumor tissues than in the control, as evidenced by immunohistochemistry. **C, D.** The expression of TIPE correlated well with p-PKM2(Ser37) in melanoma tumor tissues. **E.** Similarly, a good correlation was observed between TIPE, p-PKM2(Ser37), LDH, and CD44 in mouse xenografts. **F-I.** In addition, the expression of TIPE was positively correlated with CSCs markers, including *BMI1*, *NANOG*, *NOTCH1*, and *OCT3/4(POU5F1)* in TCGA dataset. **J.** A brief model depicting the functional impact of TIPE on metabolic reprogramming in melanoma. *** $p < 0.001$. The data represent the means $\pm$ SEM of 3 replicates.

The nuclear PKM2 routinely serves as a coactivator to transcriptionally activate certain metabolic genes (Cluntun et al., 2017 [DOI](#); Zhang et al., 2019 [DOI](#)). Because TIPE binds PKM2 and transports it into the nucleus, the dimeric form of PKM2 more easily enters the nucleus to become a coactivator of HIF-1 α , resulting in the upregulation of HIF-1 α targeted genes such as *LDHA* and *GLUT1*. Thus, we elucidate these relationships in the next step. To verify whether TIPE activates HIF-1 α transcriptional activity, we found that TIPE significantly increased the activation of the HRE promoter, especially when combined with PKM2. However, this activation was diminished by interfering with PKM2, indicating that TIPE has critical effects on the activation of HIF-1 α transcription in a PKM2-dependent manner. Because PKM2 interacts with HIF-1 α and potentiates PKM2-mediated HIF-1 α transcription, we found that TIPE increased the interaction between HIF-1 α and PKM2. In TCGA dataset, the hypoxia score was positively correlated with the mRNA expression of TIPE in melanoma, and the TIPE higher expression group had a higher hypoxia score than the lower expression group.

The mechanisms associated with PKM2 translocation are diverse, the most important of which is Erk1/2-dependent phosphorylation of PKM2 at serine 37 (Ser37) and FGFR1-dependent phosphorylation of PKM2 at tyrosine 105 (Tyr105) (Li et al., 2016 [DOI](#); Zhou et al., 2018 [DOI](#)). Consequently, we demonstrated that TIPE increased the levels of PKM2 Ser37 but not PKM2 Tyr105. Furthermore, we conjectured that this transformation caused by Ser37 phosphorylation affects the interaction between TIPE and PKM2. The results showed that mutation of PKM2 at S37A blocked its interaction with TIPE but not with Y105D. Because phosphorylation of PKM2 at serine 37 is dependent on ERK1/2 (Yang et al., 2012 [DOI](#)) and TIPE promotes AML chemoresistance by activating the ERK signaling pathway (Pang et al., 2020 [DOI](#)), we used an ERK inhibitor to further demonstrate the activation of HIF-1 α induced by TIPE. Not surprisingly, we found that TIPE facilitates the Warberg effect predominantly by driving PKM2 Ser37 phosphorylation. Furthermore, we demonstrated that the augmentation of TIPE on HRE promoter activation or HIF-1 α targeted genes such as *LDHA* and *GLUT1* was blocked by the S37A mutation, as previously reported. These results demonstrated that TIPE promotes HIF-1 α transcriptional activation via ERK-dependent PKM2 Ser37 phosphorylation.

Increasing evidence has shown that HIF-1 α promotes tumor progression, metastasis, and therapeutic drug resistance by maintaining cancer stemness (Cui et al., 2017 [DOI](#); Niu et al., 2021 [DOI](#)). Here, we observed that TIPE significantly enhanced tumorigenesis, glycolysis, and CSC properties under normoxia in melanoma, whereas suppression of PKM2 dimerization rescued these TIPE-induced effects. Interestingly, we revealed that decreasing of PKM2 dimerization can rescue TIPE induced cell proliferation, glycolysis, and CSC properties. However, this phenomenon was diminished by activation of HIF-1 α , demonstrating that TIPE promotes melanoma cells proliferation, glycolysis, and CSC properties via dimeric PKM2 dependent HIF-1 α activation. Furthermore, using a tissue chip, we showed that the expression of TIPE in melanoma tissues was dramatically increased compared to that in benign skin tumor tissues. To determine if there was a correlation between TIPE and PKM2 Ser37 levels, the results also demonstrated that TIPE expression was positively correlated with PKM2 Ser37 levels. Consistently, the expression of TIPE has a positive relationship with PKM2 Ser37, LDH, and the CSCs marker CD44 in TIPE-overexpressing cell-derived xenografts. TCGA dataset revealed that TIPE has a positive relationship with CSCs markers, including BMI1, NANOG, NOTCH1, and OCT3/4 (POU5F1) in melanoma. These results further demonstrate that TIPE maintains melanoma cell stem phenotypes in a PKM2 dimerization manner.

However, we measured metabolites such as glucose, pyruvate, and lactate at steady state, the methods such as isotope tracing experiment might be more suitable for study glucose catabolism into pyruvate, as well as tracing into lactate or into the TCA cycle following changes in TIPE expression. Moreover, the relationship between TIPE levels and melanoma patient outcome is not presented in this article. One reason is that the tissue microarray lack of the survival data. Interestingly, the TCGA dataset showed a contradictory result that the higher TIPE expression has

a favorable prognosis for melanoma. The detailed mechanisms will be discussed in our following article, and we hope that it might as a continuous research topic for TIPE in melanoma. In summary, we identified that TIPE serves as an oncogenic candidate for melanoma and determined that TIPE promotes the Warburg effect and maintains the stemness of melanoma. TIPE binds to PKM2, inducing its dimeric transformation by enhancing ERK-dependent PKM2 Ser37 phosphorylation, and thus activating the transcription of HIF-1 α , providing tumorigenicity and cancer stem-like phenomena for melanoma progression. The newly identified TIPE/PKM2/HIF-1 α axis is an ideal therapeutic target for melanoma and opens an avenue for the development of a novel strategy for precision therapy.

Materials and methods

Cell culture and plasmids construction

Human A375, A875, G361, MM96L and HEK-293T cell lines were purchased from the Shanghai Institute of Cell Biology (Chinese Academy of Sciences, Shanghai, China) and were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C under 5% CO₂. The TIPE overexpression lentiviral vector (TIPE), TIPE expression knockdown vector (TIPE-sh) and their control vectors were constructed by GeneChem company (Shanghai, China). HIF-1 α , PKM2-Flag, TIPE-HA and their truncated vectors were constructed in our laboratory. The mutant vectors such as PKM2 S37A were synthesized by Tsingke Biology Co., Ltd.(Beijing, China). All plasmids were transfected into the cells according to the manufacturer's protocol.

Cell proliferation, cell cycle and colony formation assays

Cell proliferation was measured using the Cell Counting Kit-8 method (Dojindo, Shanghai, China). For the cell cycle assay, the cells were stained with propidium iodide (PI; Sigma, USA) and were measured with a flow cytometer (FACS Aria 2, BD, USA). For the colony formation assay, cells were seeded, fixed and stained as previously described(Yang et al., 2022 [4](#)).

Transwell assay

Tranwell assay was performed as we previous described(Yang et al., 2022 [4](#)). Photographs of at least three randomly selected fields were captured and were counted.

Bioinformatics, transcriptomics and metabolomics analyses

The correlations between TIPE and HIF1A, Hypoxia score, cancer stem cells (CSCs) markers including *BMI1*, *NANOG*, *NOTCH1* and *OCT3/4(POU5F1)* were estimated by using the ASSISTANT for Clinical Bioinformation website (<https://www.aclbi.com/static/index.html> [4](#)).

Transcriptomics and metabolomics were performed and analyzed by GeneChem company (Shanghai, China). Overexpression of TIPE into G361 cells and their control group were randomly divided into three groups to perform transcriptomic analyses. In details, RNA was extracted from cells by standard extraction methods, and then the RNA samples were strictly controlled by using Nanophotometer spectrophotometer to detect RNA purity, and Agilent 2100 Bioanalyzer to detect RNA integrity. Purified mRNA by Oligo (dT) magnetic beads then randomly interrupted by divalent cations in NEB Fragmentation Buffer, and the library is built according to the NEB common library construction method. The library was qualified and processed for Illumina sequencing. Finally, the results were subjected to gene differential expression analysis. Interfering TIPE in A375 cells and its control group were randomly divided into six groups to perform metabolomic analyses. In details, samples were extracted using the methanol / acetonitrile / aqueous solution method,

followed by chromatography-mass spectrometry analysis. Finally, the data were analyzed, including univariate statistical analysis, multidimensional statistical analysis, differential metabolite screening, differential metabolite correlation analysis, KEGG pathway analysis, etc.

ATP content, ATPase activity, pyruvate kinase activity and lactic acid content measurement

The concentration of ATP was determined 72h after overexpression or interfering of TIPE by using an ATP assay kit (Beyotime, S0026) according to the manufacturer's instructions. ATPase activity was measured by using an ATPase Assay Kit (Sigma-Aldrich, MAK113). Pyruvate kinase activity and lactate production after overexpression or interfering of TIPE were measured using a Pyruvate Kinase Activity Assay Kit (Nanjing Jiancheng, A076-1-1) and Lactic Acid Assay Kit (Nanjing Jiancheng, A019-2-1) according to the manufacturer's instruction, respectively.

ECAR

The Extra Cellular Acidification Rate (ECAR) of melanoma cells were determined using the Seahorse XF extracellular flux analyzer (Agilent, Santa Clara, CA, USA). The cells were plated, washed before analysis. The cells were then treated at specific time points: glucose (10 mM), followed by oligomycin (1 μ M) and 2-DG (50 mM). Finally, the ECAR were measured by the Seahorse XF software.

Chromatin Immunoprecipitation (ChIP) Quantitative PCR (ChIP- qPCR)

Melanoma cells were cross-linked with 1% formaldehyde, lysed with sodium dodecyl sulfate buffer and sonicated. Sheared DNA was immunoprecipitated with PKM2 antibody and negative control anti-IgG, and quantified using SYBR Green Realtime PCR analysis (ABI, USA). Primer sequences are listed in **Table S5**. Fold enrichment was calculated based on Ct as $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct - Ct_{Input}$ and $\Delta(\Delta Ct) = \Delta Ct_{antibody} - \Delta Ct_{IgG}$.

Sphere formation assay

Melanoma cells transfected with TIPE or TIPE-sh were plated onto 24- well low attachment surface polystyrene plates (Corning, USA). Cells were grown in DMEM/F12 medium (Invitrogen, USA) for approximately 10 days supplemented with 20ng/mL EGF (Bioworld, BK0026), 2% B27 (Invitrogen, 17504044), 20 ng/mL basic FGF (PeproTech, #100-18B), and 1% N2(Invitrogen, 17502048).

Luciferase reporter assay

Human HEK-293T cells were seeded onto 48-well plates and transiently transfected with pHRE-Firefly luciferase reporter, TIPE-HA and PKM2- Flag or sh-RNA vector targeting PKM2 plasmids together with internal control reporter pTK (Thymidine kinase)-Renilla luciferase by Lipofectamine™ 2000 reagent (Thermo, USA). FLuc and RLuc activities were determined using the Dual-Luciferase Assay System (Promega, USA).

qPCR

mRNA was isolated and cDNAs were synthesized by using a Prime Script RT Reagent Kit (TaKaRa, China) according to manufacturer's instruction. qPCR was performed using Real SYBR Mixture (CoWin Bioscience, China) on an ABI PRISM 7500 instrument (ThermoFisher, USA). The sequences of the primers used in this study are listed in **Table S5**.

Duolink proximity ligation assay (PLA)

Duolink® In Situ PLA® kit (Sigma-Aldrich, DUO92101) was performed to demonstrate the endogenous interactions between TIPE and PKM2, and to interpret how TIPE impact the interactions between HIF-1 α and PKM2 according to we described previously (Yang et al., 2022 [DOI](#); Zhao et al., 2019 [DOI](#)). Quantification analysis was performed using the ImageJ software.

Nuclear and cytosolic fractionation

Nuclear and cytosolic fractions were prepared using NE-PER Extraction Reagents (Thermo, 78833) according to the manufacturer's recommendation. Finally, both cytosolic and nuclear subfractions were stored in -80°C prior to western blot.

Western blot

The prepared lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Bio-Rad, USA). The membranes were then blocked and incubated overnight at 4°C with the primary antibodies (as listed in **Table S6**). After incubated with secondary antibodies, the protein bands were visualized using a Tanon 5200 Imager (Shanghai, China).

Immunohistochemical and immunofluorescence

Melanoma tissue chip was purchased from Zhongke Guanghai (Xi'an, China) Intelligent Biotechnology Co., Ltd. (#K063Me01), and the clinicopathological characteristics of melanoma specimens are summarized in **Table S7**. Immunohistochemistry (IHC) staining was performed as previously reported. The samples were finally analyzed by using semiquantitative scoring criteria. The staining index values (0-12) were obtained and calculated (Yang et al., 2022 [DOI](#)). For immunofluorescence, cells were seeded onto 24-well plates at a density of 5,000 cells per well. Then, the cells were fixed using 4% formaldehyde solution and permeabilized with 0.5% Triton X-100 solution. Finally, cells were incubated overnight with antibodies prior to blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline containing 0.2% Tween-20. The fluorescence images of the cells were acquired with a fluorescence microscope equipped with appropriate filter combinations.

Co-immunoprecipitation (Co-IP) and Co-immunoprecipitation mass spectrometry (Co-IP/MS)

For Co-IP, the cells were seeded, harvested, and were then washed twice with PBS and digested and lysed in lysis buffer. The lysed supernatant was incubated with Tag-conjugated beads to bait the potential interacted proteins according to the manufacturer's instruction. For Co-IP/MS, the beads consequently were washed, eluted, resolved, and then stained with Coomassie brilliant blue. The bands were excised and digested with chymotrypsin and subjected to LC-MS/MS sequencing and data analysis by OBiO Technology Corp., Ltd. (Shanghai, China).

Flow Cytometry Analysis

Cell apoptosis was measured by using a Cell Apoptosis Analysis Kit (Engreen Biosystem Co, Ltd., Beijing, China). The cells were then incubated in serum-free medium for starvation prior to analysis and fixed using 70% ethanol for 12 h. Consequently, cells were washed, incubated with propidium iodide, and analyzed by a FACS scanner (Aria II, BD, USA). For CD44 analyzing, the cells were seeded, harvested, and incubated with anti-CD44 fluorescent antibody. Isotype-matched human immunoglobulins served as controls. Finally, the cells were counted with the FACS scanner.

Mouse xenograft tumor study

Xenograft tumor studies were conducted utilizing the 6-week-old male BALB/c nude mice purchased from the Shanghai SLAC Laboratory Animal Co., Ltd.. The total number of mice was randomly divided into respective groups and maintained in a standard pathogen free environment. All the animal experiments were approved by the Animal Ethics Committee of Zibo Central Hospital, Binzhou Medical University. Tumor growth was monitored regularly and the length, width, and height measurements taken every 7 days for 5 times. In the rescue experiment (**Fig 5E** [↗](#)), the tumor volumes were measurements taken every 5 days for 7 times. For the rescue test, one week after injection, mice bearing TIPE- vector were treated with TEPP-46. TEPP-46 (50mg/kg) or vehicle control (0.9% NaCl) was administrated via intraperitoneal injection. At the end of experiment, mice were euthanized and tumors were collected. Tumors were fixed with 4% paraformaldehyde for further immunohistochemistry (IHC) analysis.

Statistical analysis

GraphPad Prism (GraphPad Software, San Diego, CA) was used for statistical analyses. Two-tailed Student's t-tests were used to compare two groups. Linear regression analysis was explored to analyze the correlation of TIPE with other indicators. All the data are shown as the mean with standard deviations (SD) from at least three independent experiments. A *P* value of <0.05 was considered statistically significant for all tests.

Data availability Statement

All the data needed to evaluate the conclusions of the study are presented in this paper. Additional data related to this study may be requested from the corresponding author.

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Ethics approval

This study has been approved by the Medical Ethics Committee, the Animal Care and Use Committee of Zibo Central Hospital, Binzhou Medical University.

Author contributions

Peiqing Zhao: Conceptualization, Supervision. Jianxin Du: Methodology, Writing-Original draft preparation. Maojin Tian: Data curation, Investigation. Le Yang: Investigation. Ziqian Zhao: Investigation. Jigang Li: Validation. Qingqing Yin: Software. Lianqing Wang: Data curation. Wei Hu: Investigation. Yunwei Lou: Visualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest concerning this article.

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Editors

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Reviewer #1 (Public review):

Summary:

Tian et al. describes how TIPE regulates melanoma progression, stemness, and glycolysis. The authors link high TIPE expression to increased melanoma cell proliferation and tumor growth. TIPE causes dimerization of PKM2, as well as translocation of PKM2 to the nucleus, thereby activating HIF-1alpha. TIPE promotes the phosphorylation of S37 on PKM2 in an ERK-dependent manner. TIPE is shown to increase stem-like phenotype markers. The expression of TIPE is positively correlated with the levels of PKM2 Ser37 phosphorylation in murine and clinical tissue samples. Taken together, the authors demonstrate how TIPE impacts melanoma progression, stemness, and glycolysis through dimeric PKM2 and HIF-1alpha crosstalk.

The authors manipulated TIPE expression using both shRNA and overexpression approaches throughout the manuscript. Using these models, they provide strong evidence of the involvement of TIPE in mediating PKM2 Ser37 phosphorylation and dimerization. The authors also used mutants of PKM2 at S37A to block its interaction with TIPE and HIF-1alpha. In addition, an ERK inhibitor (U0126) was used to block the phosphorylation of Ser37 on PKM2. The authors show how dimerization of PKM2 by TIPE causes nuclear import of PKM2 and activation of HIF-1alpha and target genes. Pyridoxine was used to induce PKM2 dimer formation, while TEPP-46 was used to suppress PKM2 dimer formation. TIPE maintains stem cell phenotypes by increasing expression of stem-like markers. Furthermore, the relationship between TIPE and Ser37 PKM2 was demonstrated in murine and clinical tissue samples.

The evaluation of how TIPE causes metabolic reprogramming can be better assessed using isotope tracing experiments and improved bioenergetic analysis.

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Reviewer #2 (Public review):

In this article, Tian et al present a convincing analysis of the molecular mechanisms underpinning TIPE-mediated regulation of glycolysis and tumor growth in melanoma. The authors begin by confirming TIPE expression in melanoma cell lines and identify "high" and "low" expressing models for functional analysis. They show that TIPE depletion slows tumour growth in vivo, and using both knockdown and over expression approaches, show that this is associated with changes in glycolysis in vitro. Compelling data using multiple independent approaches is presented to support an interaction between TIPE and the glycolysis regulator PKM2, and over-expression of TIPE promoted nuclear translocation of PKM2 dimers. Mechanistically, the authors also demonstrate that PKM2 is required for TIPE-mediated activation of HIF1a transcriptional activity, as assessed using an HRE-promoter reporter assay, and that TIPE-mediated PKM2 dimerization is p-ERK dependent. Finally, the dependence of TIPE activity on PKM2 dimerization was demonstrated on tumor growth in vivo and in regulation of glycolysis in vitro, and ectopic expression of HIF1a could rescue inhibition of PKM2 dimerization in TIPE overexpressing cells and reduced induction of general cancer stem cell markers, showing a clear role for HIF1a in this pathway.

The detailed mechanistic analysis of TIPE mediated regulation of PKM2 to control aerobic glycolysis and tumor growth is a major strength of the study and provides new insights into the molecular mechanisms that underpin the Warburg effect in melanoma cells. The main conclusions of this paper are well supported by data, however further investigation of a

potential oncogenic effect of TIPE in melanoma patients is warranted to support the tumor promoting role of TIPE identified in the experimental models. Analysis of patient samples showed a significant increase in TIPE protein levels in primary melanoma compared to benign skin tumours, and a further increase upon metastatic progression. Moreover, TIPE levels correlate with proliferation (Ki67) and hypoxia gene sets in the TCGA melanoma patient dataset. However, the authors note in the discussion that high TIPE expression associates with better survival outcomes in the TCGA melanoma patients and these data should be included in this paper. Further investigation of how TIPE-mediated regulation of glycolysis contributes to melanoma progression is warranted to confirm the authors claims of a potential oncogenic function. Regardless, the new insights into the molecular mechanisms underpinning TIPE-mediated aerobic glycolysis in melanoma are convincing and will likely generate interest in the cancer metabolism field.

<https://doi.org/10.7554/eLife.92741.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

Summary:

Tian et al. describe how TIPE regulates melanoma progression, stemness, and glycolysis. The authors link high TIPE expression to increased melanoma cell proliferation and tumor growth. TIPE causes dimerization of PKM2, as well as translocation of PKM2 to the nucleus, thereby activating HIF-1alpha. TIPE promotes the phosphorylation of S37 on PKM2 in an ERK-dependent manner. TIPE is shown to increase stem-like phenotype markers. The expression of TIPE is positively correlated with the levels of PKM2 Ser37 phosphorylation in murine and clinical tissue samples. Taken together, the authors demonstrate how TIPE impacts melanoma progression, stemness, and glycolysis through dimeric PKM2 and HIF-1alpha crosstalk.

Strengths:

The authors manipulated TIPE expression using both shRNA and overexpression approaches throughout the manuscript. Using these models, they provide strong evidence of the involvement of TIPE in mediating PKM2 Ser37 phosphorylation and dimerization. The authors also used mutants of PKM2 at S37A to block its interaction with TIPE and HIF-1alpha. In addition, an ERK inhibitor (U0126) was used to block the phosphorylation of Ser37 on PKM2. The authors show how dimerization of PKM2 by TIPE causes nuclear import of PKM2 and activation of HIF-1alpha and target genes. Pyridoxine was used to induce PKM2 dimer formation, while TEPP-46 was used to suppress PKM2 dimer formation. TIPE maintains stem cell phenotypes by increasing the expression of stem-like markers. Furthermore, the relationship between TIPE and Ser37 PKM2 was demonstrated in murine and clinical tissue samples.

Weaknesses:

The evaluation of how TIPE causes metabolic reprogramming can be better assessed using isotope tracing experiments and improved bioenergetic analysis.

Thank you very much for your suggestions. Unfortunately, we cannot complete the isotope tracing experiments due to the lack of instruments, nor with the help of the company after consulting several companies. We are very sorry for this imperfect experiment, and we have

discussed this disadvantage in our manuscripts. Moreover, due to our negligence, there was only three metabolites were presented in the previous manuscripts. However, we have performed the routine untargeted metabolomics to demonstrate how TIPE causes metabolic reprogramming. We have added the detailed results as a new figure named as Figure S3, in which, the glycolysis pathway particularly pyruvate and lactic acid is decreased after TIPE interference.

Reviewer #2 (Public Review):

In this article, Tian et al present a convincing analysis of the molecular mechanisms underpinning TIPE-mediated regulation of glycolysis and tumor growth in melanoma. The authors begin by confirming TIPE expression in melanoma cell lines and identify "high" and "low" expressing models for functional analysis. They show that TIPE depletion slows tumour growth in vivo, and using both knockdown and over-expression approaches, show that this is associated with changes in glycolysis in vitro. Compelling data using multiple independent approaches is presented to support an interaction between TIPE and the glycolysis regulator PKM2, and the over-expression of TIPE-promoted nuclear translocation of PKM2 dimers. Mechanistically, the authors also demonstrate that PKM2 is required for TIPE-mediated activation of HIF1a transcriptional activity, as assessed using an HRE-promoter reporter assay, and that TIPE-mediated PKM2 dimerization is p-ERK dependent. Finally, the dependence of TIPE activity on PKM2 dimerization was demonstrated on tumor growth in vivo and in the regulation of glycolysis in vitro, and ectopic expression of HIF1a could rescue the inhibition of PKM2 dimerization in TIPE overexpressing cells and reduced induction of general cancer stem cell markers, showing a clear role for HIF1a in this pathway. The main conclusions of this paper are well supported by data, but some aspects of the experiments need clarification and some data panels are difficult to read and interpret as currently presented.

The detailed mechanistic analysis of TIPE-mediated regulation of PKM2 to control aerobic glycolysis and tumor growth is a major strength of the study and provides new insights into the molecular mechanisms that underpin the Warburg effect in cancer cells. However, despite these strengths, some weaknesses were noted, which if addressed will further strengthen the study.

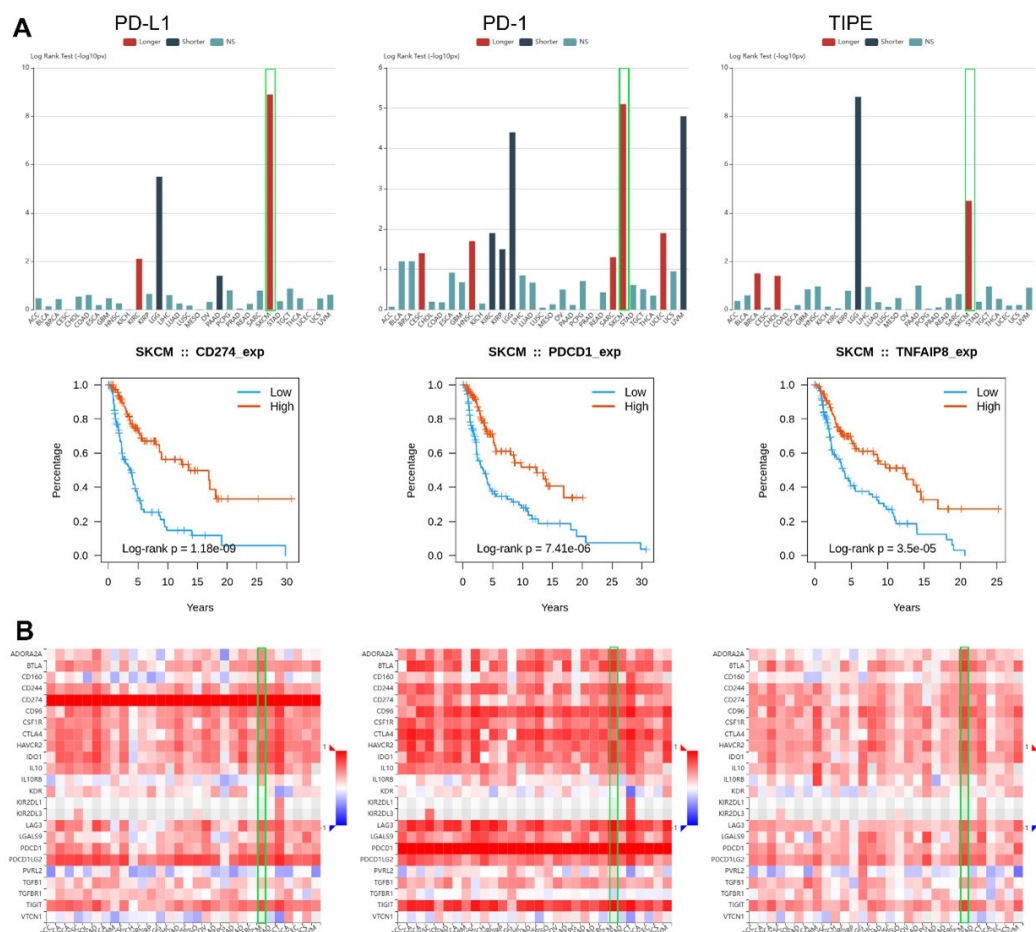
(1) The analysis of patient samples should be expanded to more directly measure the relationship between TIPE levels and melanoma patient outcome and progression (primary vs metastasis), to build on the association between TIPE levels and proliferation (Ki67) and hypoxia gene sets that are currently shown.

Thanks for your suggestions, we have added the relationship between TIPE levels and progression (non-lymph node metastasis vs lymph node metastasis). In addition, we added the association between TIPE and Ki67 or LDH levels as your advised, as shown in Figure 7.

However, the relationship between TIPE levels and melanoma patient outcome is not presented in this article. One reason is that the tissue microarray lack of the survival data. Interestingly, the TCGA dataset showed that the higher TIPE expression has a favorable prognosis for melanoma. We are also very curious about this. Our following study indicated that TIPE might serve as a positive regulator of PD-L1. Therefore, the higher expression of TIPE presents more sensitive tendency to immunotherapy, resulting in a favorable prognosis in melanoma. The detailed mechanisms will be discussed in our following article, and we hope that it might as a continuous research topic for TIPE in melanoma.

We just only disclose a little information that TIPE has a similar survival and immune signature to PD-L1 and PD-1 in melanoma as following:

Author response image 1.



(2) The duration of the in vivo experiments was not clearly defined in the figures, however, it was clear from the tumor volume measurements that they ended well before standard ethical endpoints in some of the experiments. A rationale for this should be provided because longer-duration experiments might significantly change the interpretation of the data. For example, does TIPE depletion transiently reduce or lead to sustained reductions in tumor growth?

Thanks for your suggestions. Actually, we have performed a pre-experiment before the formal experiments, and all the time points were referred to this. Furthermore, we have added the detailed time points into the figure legends as you suggested.

(3) The analysis of general cancer stem cell markers is solid and interesting, however inclusion of neural crest stem cell markers that are more relevant to melanoma biology would greatly strengthen this aspect of the study.

Thanks for your advices. We have selected two neural crest stem cell markers including Nestin and Sox10 to test their expression after overexpression of TIPE in G361 cells or interference of TIPE in A375 cells.

(4) The authors should take care that all data panels are clearly readable in the figures to facilitate appropriate interpretation by the reader.

Thanks for your suggestions. We have amended the data panels according to your advises to ensure it is clear and professionally presented.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

Major points

(1) In Figure 1D, glucose, pyruvate, and lactate were measured at a steady state. However, metabolites at steady state do not accurately depict changes in pathway activity. An isotope tracing experiment (i.e., using labelled ^{13}C glucose) can be used to study glucose catabolism into pyruvate, as well as tracing into lactate or into the TCA cycle following changes in TIPE expression. In addition, although the authors point towards changes in metabolic reprogramming, only three metabolites were measured. The use of isotope tracing to monitor metabolites from more than one pathway would be suggested to support the claim that metabolism is being reprogrammed due to TIPE.

Thank you very much for your suggestions. Unfortunately, we cannot complete the isotope tracing experiments due to the lack of instruments, nor with the help of the company after consulting several companies. We are very sorry for this imperfect experiment, and we have discussed this disadvantage in our manuscripts. Moreover, due to our negligence, there was only three metabolites were presented in the previous manuscripts. However, we have performed the routine untargeted metabolomics to demonstrate how TIPE causes metabolic reprogramming. We have added the detailed results as a new figure named as Figure S3, in which, the glycolysis pathway particularly pyruvate and lactic acid is decreased after TIPE interference.

(2) In Figure 1H, extracellular acidification was used to determine glycolytic activity. However, bicarbonate secretion can also greatly affect pH, and should be considered (PMID 25449966). Although total ATP content was measured, the contribution of ATP from glycolysis can be also determined (see PMID 28270511) to provide a more accurate representation of glycolytic ATP production.

Thanks for your suggestions again. As described at the above, we will improve our measurement methods in the future, and we have discussed our weakness in the manuscripts.

(3) On page 5, lines 108-111, the authors show that "This process represents an important regulator of the TIPE family switching between oxidative phosphorylation and aerobic glycolysis, paving the way for cancer-specific metabolism in response to low-oxygen challenge." However, there is no data on oxidative phosphorylation. What is the effect of TIPE on oxygen consumption?

Thanks for your careful and professional advices. We have conducted a thorough review of the manuscript for language accuracy and corrected this term to eliminate confusion and ensure the text is clear and professionally presented.

Minor points

(1) On page 3, line 68, it is unclear what is increasing lactate levels, as lactate can be transported inside of cells.

Thanks for your suggestions, we have corrected this misdescription to improve the overall quality and readability of the manuscript.

(2) In Figure 1B, RNA sequencing was performed on TIPE overexpressing G361 cells. The "ribosome" pathway has the highest count and lowest p-value. However, there is no mention of this in the text.

Thanks for your suggestions, we selected aerobic glycolysis as our major story comprehensively according to the transcriptomics, metabolomics and the Co-IP/MS results. Anyway, the "ribosome" pathway as you pointed might be our next research topic in the future.

(3) It would be helpful to include the cell line in Figure S1B-C as well as in the figure legend.

Thanks for your suggestions, we have added the cell line into Figure S1B-C as well as in the figure legend.

(4) Concerning supplementary figures, it would be helpful to include the panel numbers when referring to them in the main text (see line 120 or 122 as an example).

Thanks for your suggestions, we have added the panel numbers when referring to them in the main text.

(5) The sentence on lines 127-131 is very confusing.

Thanks for your suggestions, we have corrected the improper descriptions as you mentioned.

(6) In Figure S3, qPCR is misspelled in the figure legend. Also, it would be helpful to include what is meant by "relative expression" on the y-axis of Figure S3A.

Thanks for your suggestions, we have corrected the errors as you pointed. Due to the y-axis represents the expression both of TIPE and HIF-1 α , the present description might be more suitable.

(7) There is an extra space on line 196.

Thanks for your suggestions, we have corrected as you pointed.

(8) In Figure 7E LDH staining was performed. Which isoform of LDH was detected?

Actually, we stained total LDH in Figure 7E.

(9) On line 931, Warburg is misspelled.

Thanks for your suggestion, we have corrected all mentioned typos, including "Warburg" in lines 931.

Reviewer #2 (Recommendations For The Authors):

Major comments:

- Supplementary Figure 2G. Unit of time measurement for tumor growth panel needs to be defined. If this refers to days, 5 days is a relatively short period to assess tumor growth differences in vivo, and indeed, 1000-1200mm³ is a standard ethical end-point for these types of models, and this experiment was concluded well before reaching these tumor sizes. Can the authors explain why they ended this experiment at this timepoint?

Thanks for your suggestions. As you suggested, we have added the detailed time points into the figure legends. Actually, we have performed a pre-experiment before the formal experiments, and all the time points were referred to this.

- Supplementary Figure 2j - Correlation analysis between TIPE expression and overall survival outcome in melanoma patients is more relevant to support the experimental observations described in the paper than the correlation with Ki67. This analysis should also be provided. In addition, is there any difference in TIPE expression between primary and metastatic melanoma patients which would then more directly link TIPE with melanoma progression in patients?

The relationship between TIPE levels and melanoma patient outcome is not presented in this article. One reason is that the tissue microarray lack of the survival data. Interestingly, the TCGA dataset showed that the higher TIPE expression has a favorable prognosis for melanoma. We are also very curious about this. Our following study indicated that TIPE might serve as a positive regulator for PD-L1. Therefore, the higher expression of TIPE presents more sensitive tendency to immunotherapy, resulting in a favorable prognosis in melanoma. The detailed mechanisms will be discussed in our following article, and we hope that it might as a continuous research topic for TIPE in melanoma.

Furthermore, we have added the relationship between TIPE levels and progression (non-lymph node metastasis vs lymph node metastasis), and Ki67 in Figure 7.

- Figure 2 - The A2 domain protein represents a substantial reduction in the size of PKM2, which would likely have other structural effects that could affect interactions with TIPE. This should be discussed by the authors because, in this reviewer's opinion, the data presented do not shed light on the specific TIPE domain requirements for the interaction with PKM2.

Thanks for your suggestions. We have discussed this phenomenon in our manuscripts.

- Figure 4: The authors show that PKM2 recruitment to the promoters of GLUT1 and LDHA is induced by TIPE expression. Is HIF1a recruitment also induced by TIPE? This is a key gap in the detailed molecular analysis provided by the authors.

Thanks for your suggestions. This phenomenon you mentioned is very interesting, however, the expression of GLUT1 and LDHA was completely decreased when we overexpression of TIPE and PKM2 (S37A) compared to overexpression of TIPE and wild PKM2. Therefore, we believe that the higher expression of GLUT1 and LDHA was primarily promoted by TIPE-induced PKM2 recruitment.

- Figure 6: The authors present nice data for general pluripotency/stem cell markers however given melanocytes arise from the neural crest, and neural crest markers are expressed during melanoma initiation and response to therapies, analysis of neural crest stem cell markers would be appropriate to include in this analysis. For example, Sox10, Pax3, NGFR, and AQP2 have all been identified as neural crest stem cell markers expressed in both melanoma patients and experimental models.

Thanks for your advices. We have selected two neural crest stem cell markers including Nestin and Sox10 to test their expression after overexpression of TIPE in G361 cells or interference of TIPE in A375 cells.

Minor comments:

- All Figure and Supplementary Figure legends should indicate how many replicate experiments the data represents, and all error bars should be defined (StDev vs SEM).

We have added as you suggested.

- Supplementary Figure S1C - can the authors confirm the densitometry values on the western, as the band looks to be considerably larger than 1.6 fold higher compared to the control?

We redone the densitometry measurement by ImageJ. However, the result still the same.

- FACS panels in Supplementary Figure 2C-D are unreadable and should be enlarged.
 - Supplementary Figure S2i - quantification of Ki67 images appears warranted.
 - Supplementary Figure S2j - The text in the figure panel is too small and needs to be increased so the data can be interpreted accurately. Also, the authors should confirm the data is specifically from melanoma patients in the figure legend.

We have improved the quality of the figures and revised their descriptions for greater clarity and coherence, ensuring that they effectively highlight the key results of our study.

- Figure 1A - text on the heat map cannot be read. Gene-level information can be removed, and sample labels should be made larger. In panel D, no statistical analysis is shown for the metabolomics analysis. These should be added, or the authors should modify the text when referring to these data.

We have improved the quality of the figures and revised their descriptions for greater clarity and coherence, ensuring that they effectively highlight the key results of our study.

- Line 127: RNAseq data does not indicate a change in metabolites; text should be changed to say "TIPE dramatically promoted expression of genes..."

We have corrected as you suggested.

- Supplementary Figure S3c - Labels and correlation values are not readable.
 - Figure 2A - The text and details in the figure are difficult to read.
 - Figure S4 D-H - text in figure panels too small to read.

Thank you for above three questions, we have carefully reviewed the entire document to ensure all figures are clear and correctly cited, preventing any confusion and maintaining the integrity of our research findings.

- Figure 3 - the legend restates the major observations and interpretations of the figure, however does not contain enough information about what the data represents or how it was generated. The interpretation of the data should be made in the main text. For example, in panel 3. F-G the number of individual cells quantified for the analysis should be stated. In addition, given the data are generated from two completely independent cell lines, it would be more appropriate to have separate graphs for the A375 cells and G361 cells. The signal levels in the respective controls at baseline are very different, and plotted together without clear labels, making the reader question the validity of the data when this just reflects different basal signals in different cell models.

We have separated the graphs for the A375 cells and G361 cells.

| - *Figure 4 B-C - IgG controls are missing in Co-IP experiments.*

We have added the IgG controls as you suggested.

| - *Figure 5F - The unit of measure of time should be indicated on the axes; is this days?*

We measured the tumor volumes for 7 times every 5 days. We have added the detailed description in the materials and methods section.

| - *Line 348: error in text, mammosphere which should presumably be tumorsphere if from melanoma cells.*

Thanks for your suggestions, we have corrected this term to "tumorsphere" and conducted a thorough language and grammar review of the manuscript to ensure its professional presentation.

| - *Methods: more experimental details for the transcriptomic, mass spec, and metabolomics studies should be provided. There are insufficient details if readers wish to repeat these experiments.*

Thanks for your suggestions, we have corrected as you advised.

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