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Cancer Biology

PA28γ promotes the malignant progression of tumor by elevating mitochondrial function via C1QBP

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eLife Assessment

This work attempts to demonstrate an ATP-independent non-canonical role of proteasomal component PA28γ in the promotion of oral squamous cell carcinoma growth, migration, and invasion. Although the authors have addressed some concerns, uncertainties regarding the PA28g-C1QBP direct interaction still exist. The overall findings of the manuscript are **useful**, but the validation evidence is **incomplete**.

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Abstract

Proteasome activator 28γ (PA28γ) plays a critical role in malignant progression of various tumors, however, its role and regulation are not well understood. Here, using oral squamous cell carcinoma (OSCC) as main research model, we discovered that PA28γ interacted with complement 1q binding protein (C1QBP), which is dependent on the N-terminus of C1QBP rather than the known functional domain (amino acids 168-213). Notably, we found that PA28γ enhances C1QBP protein stability in OSCC. Functionally, PA28γ contributes to the malignant progression of OSCC by affecting mitochondrial morphology and oxidative phosphorylation (OXPHOS) through C1QBP in vitro and vivo. Mechanically, PA28γ upregulates the expression of optic atrophy 1 (OPA1), mitofusin 1 (MFN1), mitofusin 2 (MFN2), and the mitochondrial respiratory complex via C1QBP. Moreover, in a clinical cohort of OSCC patients, PA28γ was positively correlated with C1QBP expression and negatively correlated with prognosis. Therefore, C1QBP represents a potential therapeutic target for cancer treatment and prognosis.

Introduction

Proteasome activator 28γ (PA28γ), also known as REGγ or PSME3, is a well-known non-ATP-dependent proteasome regulator. PA28γ is overexpressed in several malignant tumors, including thyroid, colon, and breast cancer, and is significantly associated with poor prognosis (Mao, Liu, Li, & Luo, 2008; Stadtmueller & Hill, 2011). By regulating the stability of key components in various complex signaling pathways, PA28γ influences the biological behavior of tumor cells. Our previous studies revealed that the expression level of PA28γ in oral squamous cell carcinoma (OSCC) cancer nest tissues is positively correlated with patient prognosis (J. Li et al., 2015; S. Liu et al., 2018). However, its role and regulation are not well understood.

OSCC is the most common oral and maxillofacial malignant tumor(Siegel, Miller, Wagle, & Jemal, 2023; Sung et al., 2021). The pathogenic factors of OSCC include smoking, alcohol consumption, and viral infections, such as human papillomavirus (HPV). Therapies for OSCC include surgery, radiotherapy, chemotherapy and immunotherapy. Despite the rapid development of imaging, surgery, radiotherapy and immunotherapy in recent years, along with the emphasis on personalized treatment and multidisciplinary cooperation in the treatment of OSCC, the overall survival rate of OSCC patients has not significantly improved (Peres et al., 2019). The main reasons for unsatisfactory treatment efficacy include invasion of surrounding tissue, early lymph node metastasis and a tendency toward recurrence (Lindemann, Takahashi, Patel, Osman, & Myers, 2018). Metabolic reprogramming is a crucial characteristic of tumors, closely associated with malignant behaviors such as tumor growth, invasion, metastasis, and immune escape (Ohshima & Morii, 2021). Current research on metabolic reprogramming in OSCC primarily focused on mechanism of glycolytic metabolism and metabolic shift from glycolysis to oxidative phosphorylation (OXPHOS) of oral squamous cell carcinoma, which lays the groundwork for novel therapeutic interventions to counteract OSCC (Chen et al., 2024; Zhang et al., 2020). Understanding the molecular events that lead to the occurrence and development of OSCC can aid in developing new tumor-targeted therapies, which hold significant clinical application potential.

The complement 1q binding protein (C1QBP) is a crucial protein for maintaining mitochondrial function, particularly in mitochondrial OXPHOS (Ghebrehiwet, Geisbrecht, Xu, Savitt, & Peerschke, 2019; Q. Wang et al., 2022). OXPHOS is essential for the production of adenosine triphosphate (ATP), which is necessary for tumor development. C1QBP has been shown to play a significant role in cancer progression, as influencing tumor growth, invasion and metastasis (Bai et al., 2019; Hou, Lu, Wang, & Yang, 2022; Vendramin et al., 2018). Due to these roles, C1QBP presents a promising therapeutic target for various tumors, including melanoma, breast cancer, and colorectal cancer (Matsumoto & Bay, 2021). In OSCC, enhanced mitochondrial OXPHOS function might also be linked to malignant tumor progression (Vyas et al., 2021; Xiao et al., 2021; Zhu, Liu, Wu, & Wang, 2021), although the underlying mechanism remain unclear.

In this study, we discovered that PA28γ interacts with C1QBP and that PA28γ can stabilize C1QBP. This interaction enhances OXPHOS and promotes the growth, migration and invasion of OSCC cells. Additionally, the expression of PA28γ and C1QBP is increased and positively correlated in OSCC. Therefore, PA28γ and C1QBP are potential targets for the treatment and prognosis of cancer. It is the first study to describe the undiscovered role of PA28γ in promoting the malignant progression of OSCC by elevating mitochondrial function, providing new clinical insights for the treatment of OSCC.

Results

PA28γ interacts with and stabilizes C1QBP

To explore how PA28γ promotes OSCC progression, we conducted a gene–gene interaction analysis using the GeneMANIA database. This analysis identified C1QBP as a significant gene within the interaction network, with the protein-coding gene of PA28γ also playing a key role in the C1QBP interaction network (**Fig. 1A** and Appendix **Fig. 1A**). Subsequently, pull-down and coimmunoprecipitation analysis demonstrated that both endogenous and exogenous PA28γ and C1QBP could bind to each other (**Fig. 1B, C** and Appendix **Fig. 1** B, C). Moreover, the interaction of endogenous PA28γ and C1QBP was also proved by proximity ligation assay (PLA) (**Fig. 1D**). Then, we developed four OSCC cell lines with stable overexpression of PA28γ using a Flag-PA28γ-expressing lentivirus and observed that the protein level of C1QBP was higher in these cell lines compared to control cells (**Fig. 1E**). Notably, PA28γ upregulated C1QBP protein levels in a dose-dependent manner (**Fig. 1F**). To determine whether the upregulated C1QBP was due to increased stability, cells were pretreated with cycloheximide (CHX) to inhibit protein synthesis. As a result, in the presence of PA28γ, C1QBP exhibited a significantly lower turnover rate compared to the control group (**Fig. 1G, H**). In addition, PA28γ was able to stabilize C1QBP protein levels even in the presence of the proteasome inhibitor MG-132 (Appendix **Fig. 1D**). Furthermore, pull-down analysis and structure prediction of biomolecular interactions with AlphaFold 3 revealed that the PA28γ-C1QBP interaction depends on the N-terminus of C1QBP, rather than the known functional domain (amino acids 168-213) that bind to mitochondrial antiviral proteins (Peerschke et al., 2020; Xu, Xiao, Liu, Ren, & Gu, 2009) (**Fig. 1I-K** and Appendix **Fig. 1E-I**). Therefore, our results illustrated that PA28γ could interacts with C1QBP, in a manner dependent on the N-terminus of C1QBP, and that PA28γ could stabilizes C1QBP.

PA28γ and C1QBP colocalize in mitochondria and affect mitochondrial functions

To further investigate the physical interaction between PA28γ and C1QBP, we conducted an immunofluorescence (IF) assay in two OSCC cell lines, as shown these two proteins were colocalized in the mitochondria (**Fig. 2A**). Remarkably, transmission electron microscopy (TEM) images revealed fewer mitochondrial vacuoles and higher mitochondrial ridge density in PA28γ-overexpressing cells compared to control cells (**Fig. 2B** and Appendix **Fig. 2A**). IF analysis also showed increased mitochondrial lengths and areas in the PA28γ-overexpressing cells (**Fig. 2C, D**). Following that, we conducted a Cell Mito Stress Test to measure the oxygen consumption rate (OCR) (**Fig. 2E, F**). The results indicated significantly higher basal respiration, maximal OCRs and ATP production in PA28γ-overexpressing cells compared to control cells (**Fig. 2G-I** and Appendix **Fig. 2B-D**). Considering that mitochondria are the primary site for ROS generation and that ROS play a crucial role in carcinogenesis, we measured ROS levels in OSCC cells. Consistently, ROS levels were significantly higher in PA28γ-overexpressing cells (**Fig. 2J** and Appendix **Fig. 2E**). In addition, experiments conducted in PA28γ-sh OSCC cells yielded opposite results, further confirming these conclusions (Appendix **Fig. 2F-K**).

To demonstrate the carcinogenic abilities of PA28γ and C1QBP, we injected 4MOSC2 cells, a mouse OSCC line, with or without Pa28γ overexpression under the surface of the tongue in C57BL/6 mice (Appendix **Fig. 3A**). After 10 days of normal feeding, the tumors in the Pa28γ-overexpressing groups were significantly larger than those in the control group. Furthermore, via immunohistochemical (IHC) staining revealed that that PA28γ overexpression upregulate C1QBP in vivo (**Fig. 3A-C**). Consistent with the in vitro experiments, ATP production and ROS generation were significantly increased (**Fig. 3D, E**). Additionally, analysis of OSCC xenograft

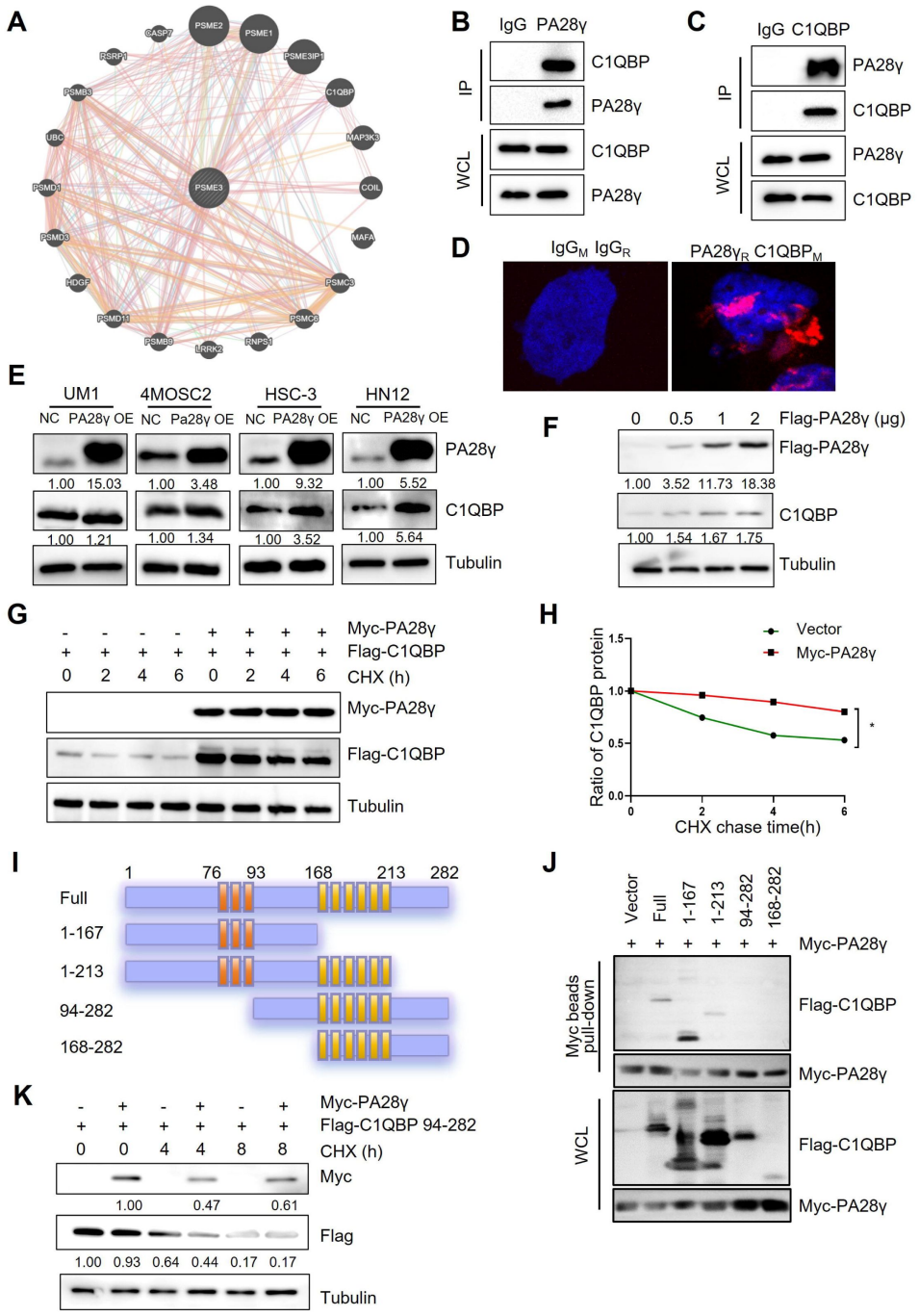

Fig. 1

The interaction between PA28γ and C1QBP.

(A) The gene network of PA28γ and C1QBP in the GeneMANIA database. **(B, C)** The interaction between endogenous PA28γ and C1QBP in HSC-3 cells was verified via immunoprecipitation. **(D)** PLA image of UM1 cells. **(E)** Western blot analysis of C1QBP in four OSCC cell lines with PA28γ-overexpressing. **(F)** Western blot analysis of C1QBP in 293T cells transfected with increasing doses of Flag-PA28γ. **(G)** 293T cells transfected with Flag-C1QBP with or without Myc-PA28γ were treated with CHX (100 μg/ml) for the indicated periods of time. **(H)** Quantification of Flag-C1QBP levels relative to tubulin levels is shown (the data are representative of 1 experiment with 3 independent biological replicates, **P*<0.05). **(I)** Full-length C1QBP and truncation with deletion of functional domains. **(J)** Pull-down of 293T cells transfected with Myc-PA28γ and full-length Flag-C1QBP or truncation mutants of functional domains for 36h. **(K)** 293T cells transfected with Flag-C1QBP 94-282 with or without Myc-PA28γ were treated with CHX (100 μg/ml) for the indicated periods of time.

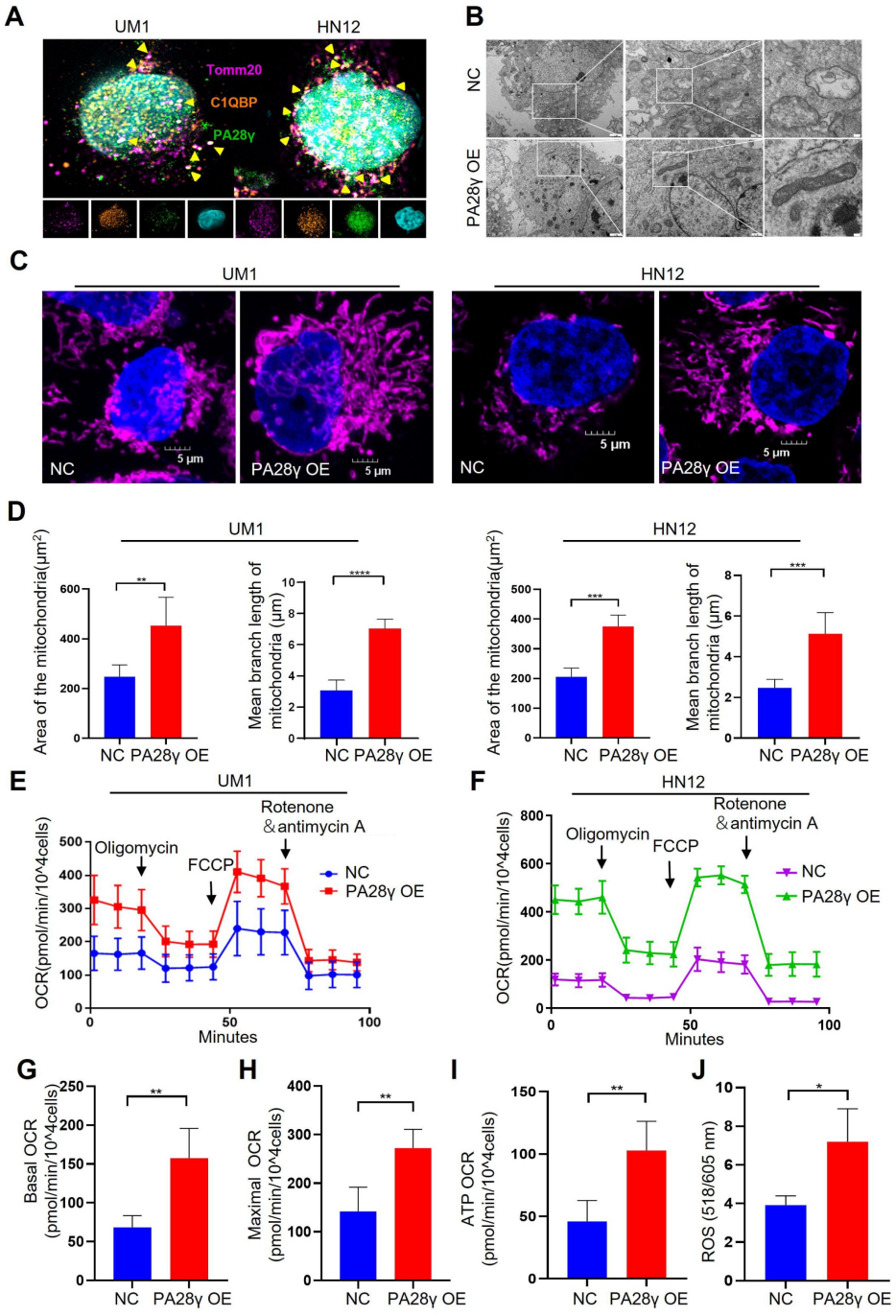

Fig. 2

PA28γ and C1QBP colocalize in mitochondria and influence mitochondrial functions *in vitro*.

(A) Confocal image of IF in two OSCC cell lines. **(B)** TEM images of PA28γ-overexpressing and control UM1 cells. **(C)** Representative confocal images of mitochondria in two OSCC cell lines. **(D)** The area and mean branch length of mitochondria in two OSCC cell lines were measured by ImageJ (the data are presented as the mean ± SD of 3 independent experiments; **P<0.01, ***P<0.001, and ****P<0.0001). **(E, F)** OCRs of PA28γ-overexpressing and control UM1 and HN12 cells were plotted using a Cell Mito Stress Test Kit (the data are presented as the means ± SDs of 3 independent experiments). **(G-I)** Basal OCRs, maximal OCRs and ATP production of PA28γ-overexpressing and control UM1 cells measured by the Cell Mito Stress Test (the data are presented as the means ± SDs of 3 independent experiments, ***P*<0.01). **(J)** ROS generation in PA28γ-overexpressing and control UM1 cells (the data are presented as the means ± SDs of 3 independent experiments; **P*<0.05).

tumor tissue in nude mice revealed that PA28γ's regulation of C1QBP in OSCC cells is independent of the immune system (Appendix **Fig. 3B, C**). In addition, we established stable Pa28γ-silenced B16 cells, a mouse melanoma cell line, and found that the level of C1qbp protein was decreased in Pa28γ-silenced B16 cells (Appendix **Fig. 3D**). Cells with or without Pa28γ silencing were grafted into the flanks of Pa28γ $^{ko/ko}$ and Pa28γ $^{wt/wt}$ C57BL/6 mice by subcutaneous injection (Appendix **Fig. 3E**). Remarkably, the tumors derived from the silenced Pa28γ cells in Pa28γ $^{ko/ko}$ mice were smaller than those in the other groups (**Fig. 3F-H**). The trend of ATP and ROS was consistent with tumor size (**Fig. 3I, J**), and the protein levels of C1qbp in the tumors were also reduced in the Pa28γ-silenced groups (**Fig. 3K**).

Collectively, these data suggest that PA28γ, which co-localizes with C1QBP in mitochondria, may involve in regulating mitochondrial morphology and function.

PA28γ regulates mitochondrial OXPHOS by upregulating C1QBP

Due to the results of targeted metabolomics analysis by mass spectrometry (Appendix **Fig. 4A, B**), it was found that PA28γ may affect the biological behavior and function of OSCC by influencing metabolism. Therefore, we explored the protein levels of the mitochondrial respiratory chain complex, C1QBP and other mitochondrial functional proteins. We found that the levels of C1QBP, complex I and IV proteins, as well as OPA1, MFN1 and MFN2 proteins, were upregulated in PA28γ-overexpressing OSCC cells (**Fig. 4A, B** and Appendix **Fig. 4C, D**), These increases could be reversed by C1QBP silencing in PA28γ-overexpressing OSCC cells (**Fig. 4C**).

Consistent with this, the OCRs of C1QBP-silenced PA28γ-overexpressing OSCC cells were markedly lower than those of control cells (**Fig. 4D** and Appendix **Fig. 4E-G**). Subsequently, we constructed a series of experiments to detect the biological behavior of control and PA28γ-overexpressing OSCC cells with or without C1QBP silencing. C1QBP knockdown significantly attenuated the migration, invasive and proliferation capabilities previously augmented by PA28γ overexpression (**Fig. 4E-H**). These data suggest that PA28γ enhances mitochondrial OXPHOS function through C1QBP.

C1QBP expression was positively associated with PA28γ expression and was associated with a worse prognosis in patients with OSCC or SKCM

To determine the correlation between PA28γ and C1QBP, IHC staining was performed in the clinical cohort of oral mucosa carcinogenesis. The results showed that both C1QBP and PA28γ were notably upregulated during the progression of oral mucosa carcinogenesis (**Fig. 5A, B** and Appendix **Fig. 5A**), and the levels of PA28γ and C1QBP were positively related (**Fig. 5C**). Similarly, both C1QBP and PA28γ were upregulated in metastatic OSCC tissues (**Fig. 5D, E** and Appendix **Fig. 5B**), and the levels of PA28γ and C1QBP were consistently positively correlated in OSCC cohort (**Fig. 5F**). Notably, consistent with our previous findings on PA28γ (J. Li et al., 2015), C1QBP or combining PA28γ and C1QBP could be a negative predator in our multicenter OSCC clinical cohort (**Fig. 5G, H**), TCGA HNSC database (**Fig. 5I, J**) and TCGA SKCM database (Appendix **Fig. 5C, D**).

Discussion

Metabolic reprogramming is one of the hallmarks of malignant tumors and is related to the malignant biological behavior of tumors (Faubert, Solmonson, & DeBerardinis, 2020; Hanahan, 2022; Tsai et al., 2023; Xia et al., 2021). The metabolic phenotype of tumor cells differs from that of normal cells and dynamically changes during tumor progression. OSCC is a common head and neck malignancy that is still a growing global health problem (Johnson et al., 2020). The

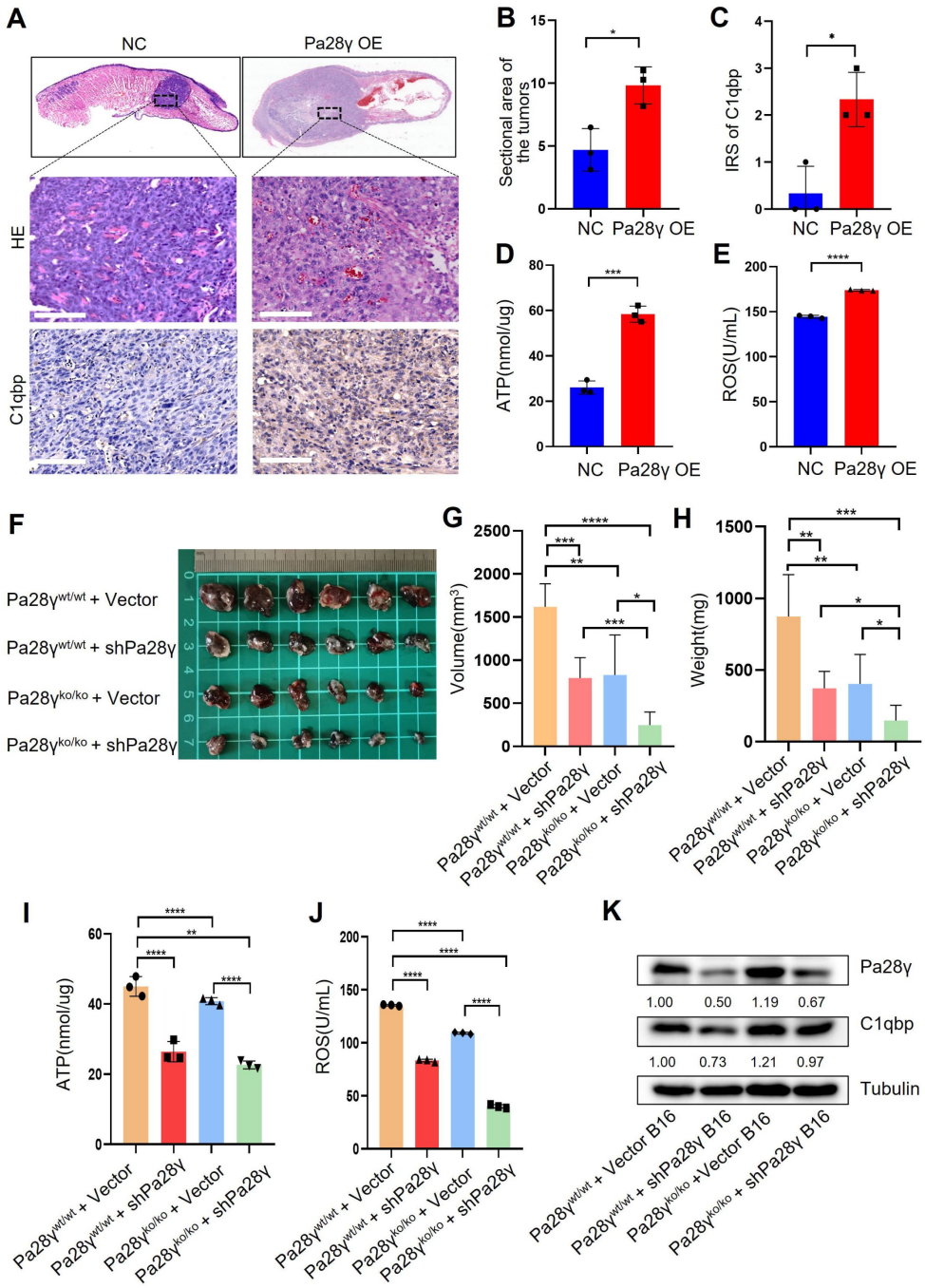

Fig. 3

PA28γ affect mitochondrial functions *in vivo*.

(A) Representative images of H&E staining and IHC staining of C1qbp of tongue sections from mice (n=3). **(B)** Quantification of the sectional area in tumors from the Pa28γ-overexpressing and control groups (n=3; the data are presented as the means ± SDs of 3 samples; **P*<0.05). **(C)** Comparison of the immunoreactive scores (IRSs) of C1qbp antibody staining in the Pa28γ-overexpressing and control groups (n=3, the data are presented as the means ± SDs; *P<0.05). **(D, E)** Quantification of ATP production and ROS levels in tumors from the Pa28γ-overexpressing and control groups (n=3; the data are presented as the means ± SDs of 3 samples; **P*<0.05, ****P*<0.001, *****P*<0.0001). **(F)** Images of the tumors in different groups at the endpoint (n=6). **(G-J)** The volume, weight, ATP and ROS of tumors in different groups at the endpoint (n=6; the data are presented as the means ± SDs of 3 independent experiments; **P*<0.05, **P<0.01, ****P*<0.001, *****P*<0.0001). **(K)** Western blot analysis of tumors in different groups.

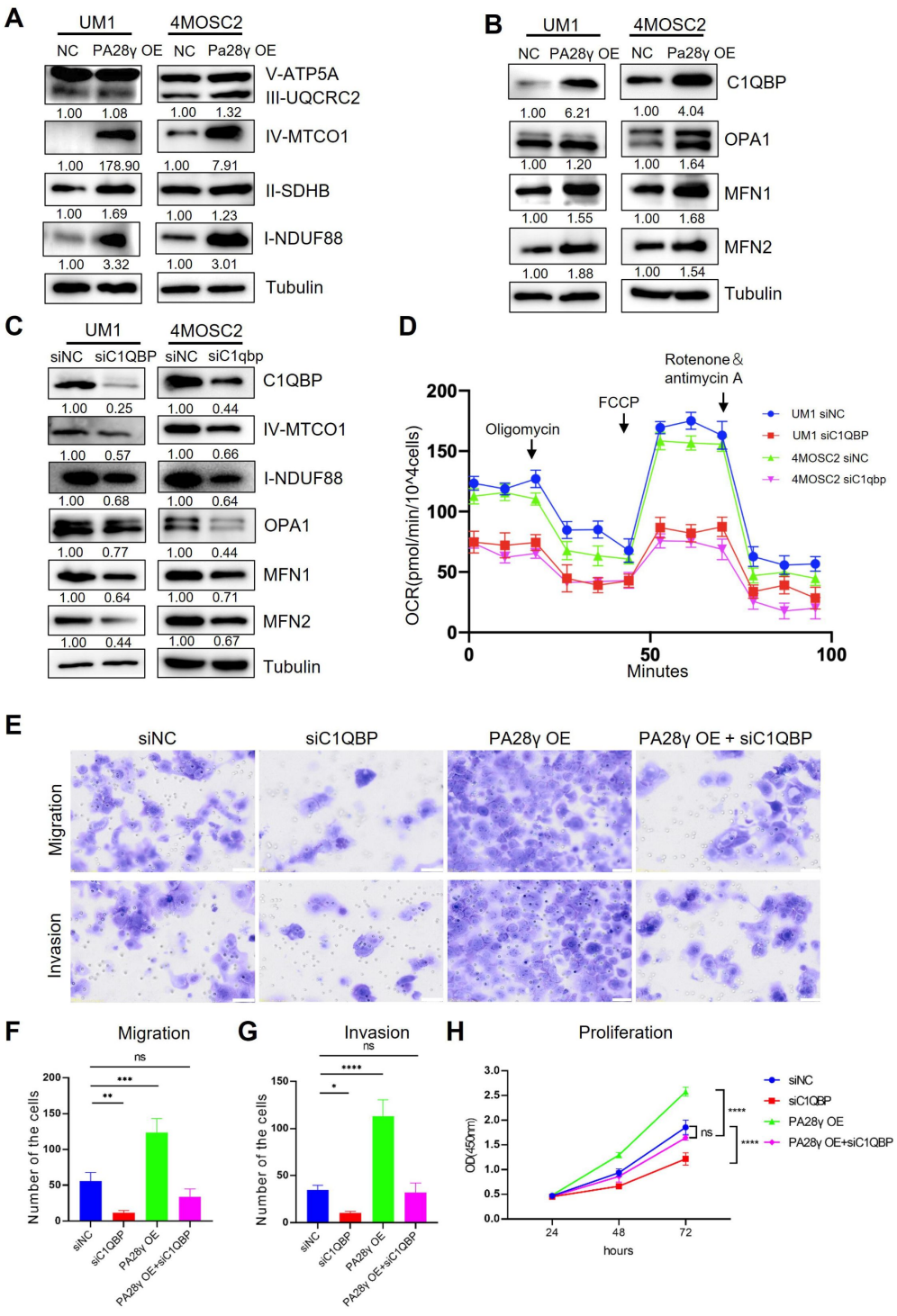

Fig. 4

PA28γ regulates mitochondrial OXPHOS and cellular biological behavior via C1QBP.

(A, B) Western blot analysis of PA28γ-overexpressing and control UM1 and 4MOSC2 cells. **(C)** Western blot analysis of PA28γ-overexpressing OSCC cells transfected with siNC or siC1QBP. **(D)** OCRs of C1QBP-silenced and control PA28γ-overexpressing UM1 and 4MOSC2 cells (the data are presented as the means ± SDs of 3 independent experiments). **(E-H)** Cell migration, invasion and proliferation in control, C1QBP-silenced, PA28γ-overexpressing and PA28γ-overexpressing + C1QBP-silenced UM1 cells (the data are presented as the means ± SDs of 3 independent experiments; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001).

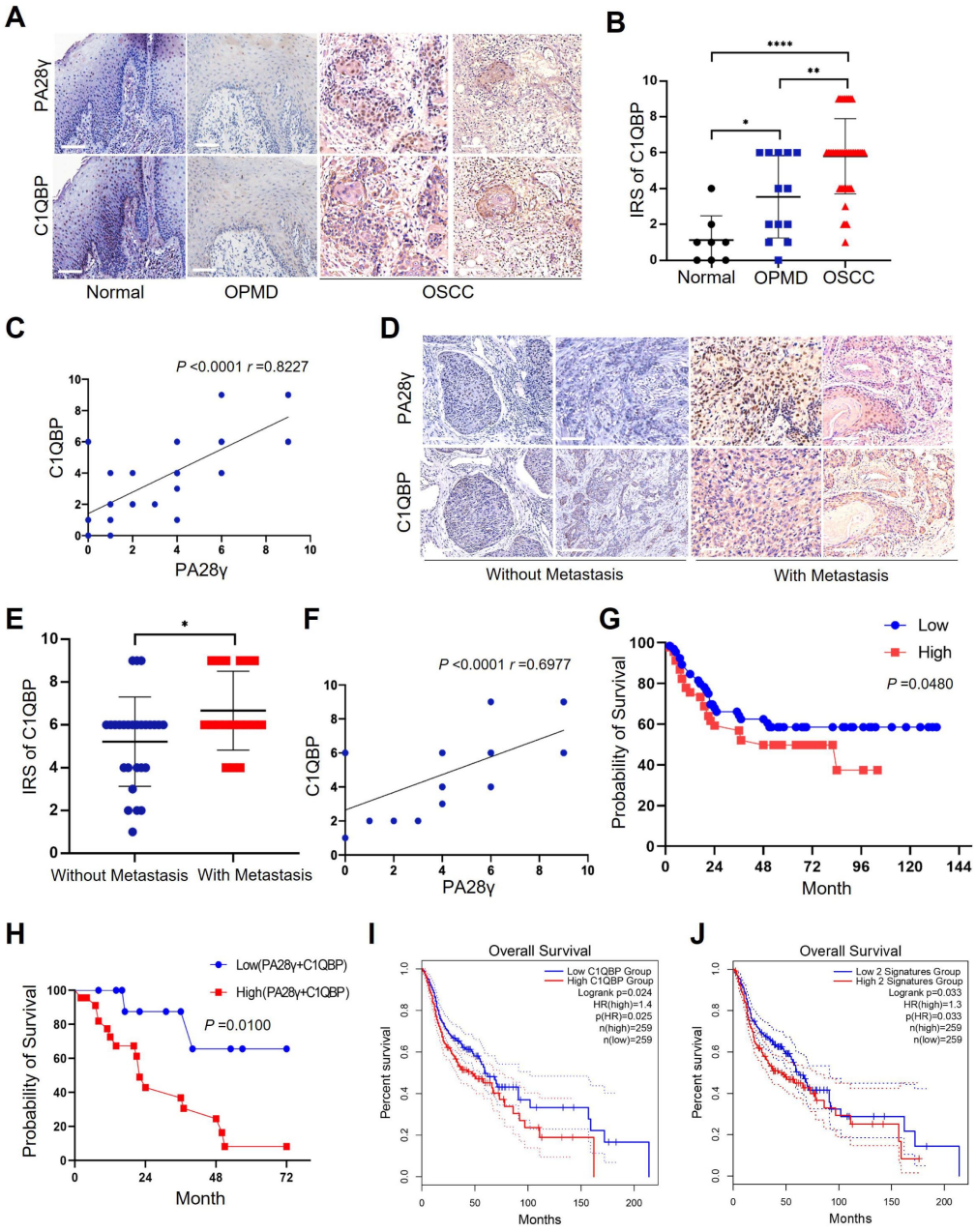

Fig. 5

The correlation between PA28γ and C1QBP in the carcinogenesis and development of OSCC.

(A) Representative IHC staining of PA28γ and C1QBP in normal (n=8), OPMD (n=13) and OSCC (n=45) samples. **(B)** Comparison of the immunoreactive scores (IRSs) of C1QBP between the normal, OPMD and OSCC groups (the data are presented as the means ± SDs; **P*<0.05, ***P*<0.01, *****P*<0.0001). **(C)** Spearman correlation analysis was used to test the correlation between PA28γ and C1QBP in normal, OPMD and OSCC tissues (*P*<0.0001, *r*=0.8227). **(D)** Representative IHC staining of PA28γ and C1QBP in nonmetastatic (n=27) and metastatic (n=18) OSCC patients. **(E)** Comparison of the IRSs of C1QBP in the nonmetastatic and metastatic OSCC groups (the data are presented as the means ± SDs; **P*<0.05). **(F)** Spearman correlation analysis was used to test the correlation between PA28γ and C1QBP in OSCC tissues (*P*<0.0001, *r*=0.6977). **(G)** Kaplan–Meier analysis of the protein expression of C1QBP in our multicenter OSCC clinical cohort (n=295, *P*=0.0480). **(H)** Kaplan–Meier analysis of both low or high protein expression of C1QBP and PA28γ in our multicenter OSCC clinical cohort (n=295, *P*=0.0100). **(I)** Kaplan–Meier analysis of the protein expression of C1QBP in TCGA HNSC database (n=259, *P*=0.025). **(J)** Kaplan–Meier analysis of both low or high protein expression of C1QBP and PA28γ in TCGA HNSC database (n=259, *P*=0.033).

development of OSCC is a complex and multifaceted process in which metabolic reprogramming appears to be important (B. Liu, Si, Wei, Zhang, & Chen, 2023). However, the mechanism of metabolic reprogramming remains unclear. Excitingly, we found the evidence that PA28γ interacts with and stabilizes C1QBP. We speculate that aberrantly accumulated C1QBP enhances the function of mitochondrial OXPHOS and leads to the production of additional ATP and ROS by activating the expression and function of OPA1, MNF1, MFN2 and mitochondrial respiratory chain complex proteins. This process results in resulting in mitochondrial fusion and malignant tumor progression (**Fig. 6**). Our study highlights the underlying mechanism by which PA28γ participates in the regulation of OXPHOS by upregulating C1QBP in OSCC.

C1QBP is also called gClqR, p32, p33 and HABP1, can be distributed inside cells, on the cell surface, and can also be secreted extracellularly (Egusquiza-Alvarez & Robles-Flores, 2022). Within cells, C1QBP is primarily located in the mitochondria. The key functions of mitochondrial C1QBP include maintaining OXPHOS, supplying energy to cells, and achieving homeostasis of mitochondrial metabolism (Ghebrehiwet et al., 2019; Q. Wang et al., 2022). C1QBP is highly expressed in cells that require a large amount of energy, such as the heart, skeletal muscle, testis, ovary, small intestine and colon (Feichtinger et al., 2017). Sufficient energy is essential for cancer development, and C1QBP promotes malignant behaviors such as tumor invasion and metastasis by enhancing OXPHOS function in various cancers (Raschdorf et al., 2021; Q. Wang et al., 2022). However, there are no reports on the expression and functions of C1QBP in OSCC. In our study, we found the evidence that PA28γ interacts with and stabilizes C1QBP. As a proteasome activator, PA28γ usually affects the development of tumors by regulating the stability of various important proteins. For example, PA28γ can promote the degradation of p53, p21 leading to cancer progression (J. Liu et al., 2010). In addition, PA28γ can also play as a non-degradome role on tumor angiogenesis. For example, PA28γ can regulate the activation of NF-κB to promote the secretion of IL-6 and CCL2 in OSCC cells, thus promoting the angiogenesis of endothelial cells (S. Liu et al., 2018). However, the function of mitochondrial PA28γ may be differ from that of nuclear PA28γ (Mao et al., 2008). Our study reveals that PA28γ interacts with C1QBP and stabilizes C1QBP at the protein level. Therefore, we speculate that the binding sites of PA28γ and C1QBP may mask the specific post-translational modification sites of C1QBP and inhibit its degradation. For example, the transcriptional coactivator p300 interacts with Smad7 by acetylating two lysine residues in its N-terminus, which stabilize Smad7 and protect it from TGFβ-induced degradation (Grönroos, Hellman, Heldin, & Ericsson, 2002). Overall, the specific mechanism involved in the interaction between PA28γ and C1QBP still needs further exploration.

Considering that C1QBP is a vital protein for maintaining mitochondrial metabolism (Tian et al., 2023), we further investigated the function underlying the malignant progression of OSCC through the interaction between PA28γ and C1QBP in vitro and in vivo. PA28γ and C1QBP colocalized in the mitochondria, and the stabilization of C1QBP by PA28γ enhanced mitochondrial morphology and OXPHOS. This enhancement is crucial for ATP production, and ATP is the primary energy currency of cellular metabolism. Therefore, PA28γ's regulation of OXPHOS may impact cellular energy metabolism. PA28γ has been reported to activate the mTORC1 signaling pathway in hepatocellular carcinoma cells to promote glycolysis and inhibit OXPHOS (Yao et al., 2021). These findings contrast with our study, potentially due to organ heterogeneity or the complexity of metabolic reprogramming.

In the tissue microenvironment of oral lichen planus, a potentially malignant oral disorder, PA28γ in epithelial cells can regulate T cell differentiation (Y. Wang et al., 2024), while PA28γ in CAFs is involved in the crosstalk between stromal cells and tumor cells (Z. Li et al., 2024). This suggests that PA28γ may interact with the tumor immune microenvironment. Our phenotypically similar OSCC xenograft tumor models in both immunocompetent and nude mice indicate that the regulation of mitochondrial OXPHOS by PA28γ localized in tumor cell mitochondria does not entirely depend on the immune system (Z. Wang et al., 2019).

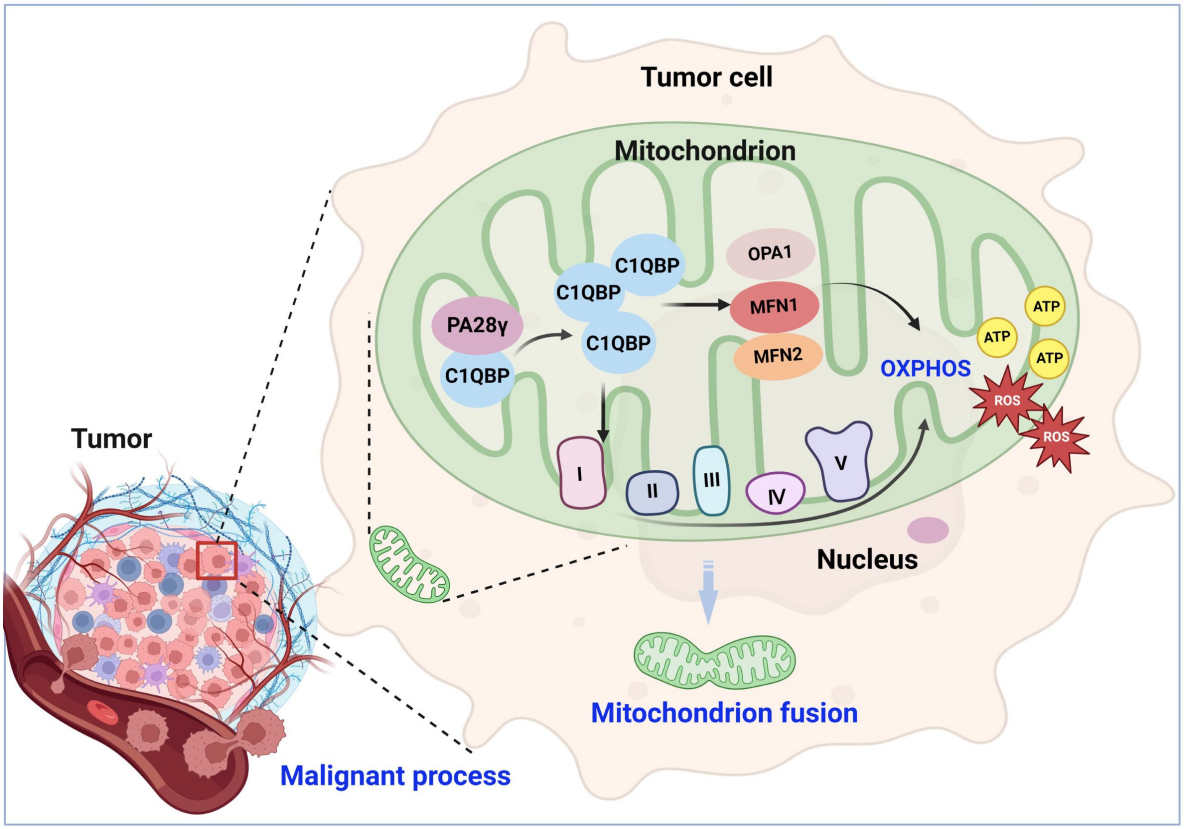

Fig. 6

Molecular mechanism through which PA28γ interacts with C1QBP in the malignant progression of tumor.

PA28γ can interacts with and stabilize C1QBP, which can activate the expression and function of OPA1, MNF1, MFN2 and mitochondrial respiratory chain complex proteins, resulting in enhanced mitochondrial OXPHOS, mitochondrial fusion and malignant tumor progression.

Furthermore, our study reveals that PA28γ can regulate C1QBP and influence mitochondrial morphology and function by enhancing the expression of OPA1, MFN1, MFN2 and the mitochondrial respiratory complex. Mitochondrial fusion, crucial for oxidative metabolism and cell proliferation, is regulated by MFN1, MFN2, and OPA1. The first two fuse with the outer mitochondrial membrane, while the last fuses with the inner mitochondrial membrane (Westermann, 2010). OPA1 is an essential inner mitochondrial membrane protein with multiple functions, including regulating mitochondrial fusion, crest morphology, mtDNA stability, and interacting with the mitochondrial respiratory chain to regulate OXPHOS (Del Dotto et al., 2018).

Finally, we analyzed the expression patterns and significance of PA28γ and C1QBP in a clinical cohort. PA28γ and C1QBP were negatively correlated with prognosis in the OSCC clinical cohort. Notably, some studies have shown that OXPHOS is upregulated in various tumors, with some advanced tumors preferring OXPHOS metabolically (Ohshima & Morii, 2021; Qiu, Li, & Zhang, 2023). OXPHOS activity is associated with the recurrence of OSCC (Noh et al., 2023). Additionally, the enhancement of OXPHOS and ATP production by ROS proto-oncogene 1, which localizes to mitochondria in OSCC, promotes the OSCC invasion(Noh et al., 2023; Grimm et al., 2014). These findings are consistent with our results. Our study not only explain the mechanisms and signaling networks underlying the oncogenic potential of PA28γ but also contribute to understanding the possible mechanisms involved in OSCC metabolic reprogramming.

In summary, we found that PA28γ could interact with C1QBP and stabilize C1QBP. This enhances the function of OXPHOS, leading to increased production of ATP and ROS by activating the expression and function of OPA1, MNF1, MFN2 and the mitochondrial respiratory chain complex. This process promotes mitochondrial fusion and malignant tumor progression. The expression of PA28γ and C1QBP was increased and positively correlated in OSCC. High expression of PA28γ and C1QBP was correlated with poor prognosis in OSCC patients, indicating that both factors could serve as potential targets for the treatment and prognosis of OSCC.

Materials and methods

Patients and Follow-up

Our study included 4 cohorts. The first cohort included 8 normal controls, 13 patients with oral potentially malignant disorder (OPMD), and 45 patients with OSCC (Appendix Table 1). These patients visited the West China Hospital of Stomatology in 2021. The normal tissues were obtained from patients who underwent maxillofacial plastic surgery. The second cohort for survival analysis included 295 patients diagnosed with primary OSCC tumors who visited West China Hospital of Stomatology, Peking University Hospital of Stomatology and Guangdong Provincial Stomatological Hospital from 2005 to 2009 (Appendix Table 2). None of the patients had cancer in other organs. These patients received regular follow-up for 1-133 months after the operation. The protocol of this study was approved by the ethics committee of the West China Hospital of Stomatology (Approval number: WCHSIRB-D-2020-046). The third cohort, comprising 518 patients with head and neck squamous cell carcinoma (HNSCC), was obtained from The Cancer Genome Atlas (TCGA) database (Appendix Table 3). The last cohort, comprising 458 patients with skin cutaneous melanoma (SKCM), was obtained from TCGA database (Appendix Table 4).

Laboratory Experiments

All animal studies were approved by the Animal Care and Use Committee for the State Key Laboratory of Oral Diseases (Approval number: WCHSIRB-D-2022-032) in compliance with the Guide for the updated Animal Research: Reporting of In Vivo Experiments (ARRIVE) 2.0 guidelines. Other methods are detailed in the *Supplemental Materials and Methods*, including cell culture and

cell-related experiments, animal models, mitochondrial function detection, western blot/immunoprecipitation assays, hematoxylin and eosin (H&E) staining, and immunohistochemistry.

Statistical Analysis

The data were analyzed using GraphPad Prism software version 8.0. The statistical data are expressed as the mean ± standard deviation (SD). Chi-square and Fisher exact tests, Spearman correlation tests, Wilcoxon rank-sum tests, one-way ANOVA and Student's t tests were used to analyze the data. The Kaplan–Meier method was used for survival analysis, and the log-rank test was used to evaluate the prognostic value of C1QBP in OSCC patients. The data are shown as the mean ± standard error, and a value of $P<0.05$ was considered to indicate statistical significance: $P<0.05$*, $P<0.01$**, $P<0.001$***, and $P<0.0001$****. Each experiment was repeated 3 times.

Acknowledgements

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Additional information

Author contributions

J. Wang, Y. Shi, X. Zeng, J. Li contributed to conception and design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; Y. Wang, Y. Shen, H Liu contributed to data acquisition; S. Sun, Y. Wang contributed to data analysis and interpretation; X. Zhou, Q. Chen contributed to conception and design, critically revised the manuscript. All the authors gave final approval and agreed to be accountable for all aspects of the work.

Additional files

Supplemental materials and methods

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Editors

Reviewer #2 (Public review):

This manuscript determines how PA28g, a proteasome regulator that is overexpressed in tumors, and C1QBP, a mitochondrial protein for maintaining oxidative phosphorylation that plays a role in tumor progression, interact in tumor cells to promote their growth, migration and invasion. Evidence for the interaction and its impact on mitochondrial form and function was provided although it is not particularly strong.

The revised manuscript corrected mislabeled data in figures and provides more details in figure legends. Misleading sentences and typos were corrected. However, key experiments that were suggested in previous reviews were not done, such as making point mutations to disrupt the protein interactions and assess the consequence on protein stability and function. Results from these experiments are critical to determine whether the major conclusions are fully supported by the data.

The second revision of the manuscript included the proximity ligation data to support the PA28g-C1QBP interaction in cells. However, the method and data were not described in sufficient detail for readers to understand. The revision also includes the structural models of the PA28g-C1QBP complex predicted by AlphaFold. However, the method and data were not described with details for readers to understand how this structural modeling was done, what is the quality of the resulting models, and the physical nature of the protein-protein interaction such as what kind of the non-covalent interactions exist in the interface of the protein complexes. Furthermore, while the interactions mediated by the protein fragments were tested by pull-down experiments, the interactions mediated by the three residues were not tested by mutagenesis and pull-down experiments. In summary, the revision was improved, but further improvement is needed

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Author response:

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

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***

> ***Comment of Review of Revised Version:***
>
> *Although the authors have partly corrected the manuscript by removing the mislabeling in their Co-IP experiments, my primary concern on the actual functional connotations and direct interaction between PA28y and C1QBP still remains unaddressed. As already mentioned in my previous review, since the core idea of the work is PA28y's direct interaction with C1QBP, stabilizing it, the same should be demonstrated in a more convincing manner.*
>
> *My other observation on the detection of C1QBP as a doublet has been addressed by usage of anti-C1QBP Monoclonal antibody against the polyclonal one used before. C1QBP doublets have not been observed in the present case.*
>
> *The authors have also worked on the presentation of the background by suitably modifying the statements and incorporating appropriate citations.*
>
> *However, the authors are requested to follow the recommendations provided to them by the reviewers to address the major concerns.*

Thank you very much for your comments. We appreciate your concerns regarding the need for more direct evidence to support the stabilizing interaction between PA28γ and C1QBP. In response to your feedback, we have taken additional steps to provide more convincing evidence of this interaction.

To complement our existing pull-down and Co-IP experiments, we utilized AlphaFold 3 to predict the three-dimensional structure of the PA28γ-C1QBP complex. The predicted model reveals specific residues and interfaces that are likely involved in the direct interaction between PA28γ and C1QBP. Our analysis indicates that this interaction may depend on amino acids 1-167 and 1-213 of C1QBP (Revised Appendix Figure 1E-H). Furthermore, aspartate (ASP), as the 177th amino acids of PA28γ, was predicted to interact with the 76th amino acid threonine (THR) and the 78th amino acid glycine (GLY) of C1QBP (Revised Appendix Figure 1I). This structural insight was further validated by our immunoprecipitation experiments (Revised Figure 1J). These findings provide a molecular basis for the observed stabilizing effect and suggest potential mechanisms by which PA28γ influences C1QBP stability. Specifically, the identified interaction sites offer clues into how PA28γ may stabilize C1QBP at the molecular level.

Furthermore, we performed proximity ligation assays (PLA) to detect in situ interactions between PA28γ and C1QBP at the single-cell level. PLA results clearly demonstrate the presence of PA28γ-C1QBP complexes within cells, providing direct evidence of their physical interaction (Revised Figure 1D). This approach overcomes some of the limitations associated with traditional IP experiments and confirms the direct nature of the interaction.

In summary, the integration of AlphaFold 3 predictions, PLA data, and our previous Pull-down and Co-IP experiments provides robust and direct evidence for a stable interaction between PA28γ and C1QBP. We believe that these additional findings significantly reinforce our conclusions and effectively address the concerns raised by the reviewers. Once again, thank you for your valuable feedback, which has been instrumental in refining and enhancing our study.

> ***Reviewer #2 (Public review):***
>
> ***Comment of Review of Revised Version:***
>
> *Weaknesses:*

Many data sets are shown in figures that cannot be understood without more descriptions either in the text or the legend, e.g., Fig. 1A. Similarly, many abbreviations are not defined.

The revision addressed these issues.

Some of the pull-down and coimmunoprecipitation data do not support the conclusion about the PA28g-C1QBP interaction. For example, in Appendix Fig. 1B the Flag-C1QBP was detected in the Myc beads pull-down when the protein was expressed in the 293T cells without the Myc-PA28g, suggesting that the pull-down was not due to the interaction of the C1QBP and PA28g proteins. In Appendix Fig. 1C, assume the SFB stands for a biotin tag, then the SFB-PA28g should be detected in the cells expressing this protein after pull-down by streptavidin; however, it was not. The Western blot data in Fig. 1E and many other figures must be quantified before any conclusions about the levels of proteins can be drawn.

The revision addressed these problems.

The immunoprecipitation method is flawed as it is described. The antigen (PA28g or C1QBP) should bind to the respective antibody that in turn should binds to Protein G beads. The resulting immunocomplex should end up in the pellet fraction after centrifugation, and analyzed further by Western blot for coprecipitates. However, the method in the Appendix states that the supernatant was used for the Western blot.

The revision corrected this method.

To conclude that PA28g stabilizes C1QBP through their physical interaction in the cells, one must show whether a protease inhibitor can substitute PA28q and prevent C1QBP degradation, and also show whether a mutation that disrupt the PA28g-C1QBP interaction can reduce the stability of C1QBP. In Fig. 1F, all cells expressed Myc-PA28g. Therefore, the conclusion that PA28g prevented C1QBP degradation cannot be reached. Instead, since more Myc-PA28g was detected in the cells expressing Flag-C1QBP compared to the cells not expressing this protein, a conclusion would be that the C1QBP stabilized the PA28g. Fig. 1G is a quantification of a Western blot data that should be shown.

The binding site for PA28g in C1QBP was mapped to the N-terminal 167 residues using truncated proteins. One caveat would be that some truncated proteins did not fold correctly in the absence of the sequence that was removed. Thus, the C-terminal region of the C1QBP with residues 168-283 may still bind to the PA29g in the context of full-length protein. In Fig. 1I, more Flag-C1QBP 1-167 was pull-down by Myc-PA28g than the full-length protein or the Flag-C1QBP 1-213. Why?

The interaction site in PA28g for C1QBP was not mapped, which prevents further analysis of the interaction. Also, if the interaction domain can be determined, structural modeling of the complex would be feasible using AlphaFold2 or other programs. Then, it is possible to test point mutations that may disrupt the interaction and if so, the functional effect.

The revision added AlphaFold models for the protein interaction. However, the models were not analyzed and potential mutations that would disrupt the interact were not predicted, made and tested. The revision did not addressed the request for the protease inhibitor.

Thank you for your insightful comments regarding the binding site of PA28γ in C1QBP. We appreciate your concern about the potential misfolding of truncated proteins and the

possible interaction between the C-terminal region (residues 168-283) of C1QBP and PA28γ in the context of full-length protein.

To address these concerns, we have conducted additional analyses and experiments to provide a more comprehensive understanding of the interaction between PA28γ and C1QBP. Using AlphaFold 3, we predicted the three-dimensional structure of the PA28γ-C1QBP complex. The model reveals specific residues and interfaces that are likely involved in the direct interaction between PA28γ and C1QBP. Notably, our structural analysis indicates that the interaction may primarily depend on amino acids 1-167 and 1-213 of C1QBP (Revised Appendix Figure 1E-H). Furthermore, aspartate (ASP), as the 177th amino acids of PA28γ, was predicted to interact with the 76th amino acid threonine (THR) and the 78th amino acid glycine (GLY) of C1QBP (Revised Appendix Figure 1I). This prediction supports the idea that the N-terminal region of C1QBP is crucial for its interaction with PA28γ. Regarding the observation in old Figure 1I (Revised Figure 1J), where more Flag-C1QBP 1-167 was pulled down by Myc-PA28γ compared to the full-length protein or Flag-C1QBP 1-213, we believe this can be explained by several factors:

A. The truncation of C1QBP to residues 1-167 may expose key interaction sites that are partially obscured in the full-length protein. This enhanced accessibility could lead to stronger binding affinity and higher pull-down efficiency.

B. While it is possible that some truncated proteins do not fold correctly, our data suggest that the N-terminal fragment (1-167) retains sufficient structural integrity to interact effectively with PA28γ. The increased pull-down of this fragment suggests that it captures the essential elements required for binding.

C. The C-terminal region (168-283) might exert steric hindrance or allosteric effects on the N-terminal binding site in the context of the full-length protein. This interference could reduce the overall binding efficiency, leading to less pull-down of full-length C1QBP compared to the truncated version.

Compared with the control group, the presence of Myc-PA28γ significantly increased the expression level of Flag-C1QBP (r Revised Figure 1G). Gray value analysis showed that in cells transfected with Myc-PA28γ, the decay rate of Flag-C1QBP was significantly slower than that of the control group (Revised Figure 1H), suggesting that PA28γ can delay the protein degradation of C1QBP and stabilize its protein level. This indicates that an increase in the level of PA28γ protein can significantly enhance the expression level of C1QBP protein, while PA28γ can slow down the degradation rate of C1QBP and improve its stability. In addition, our western blot analysis also proved that PA28γ could still prevent the degradation of C1QBP under the action of proteasome inhibitor MG-132 (Revised Appendix Figure 1D). Moreover, PA28γ could not stabilize the mutation of C-terminus of C1QBP (amino acids 94-282), which was not the interaction domain of PA28γ-C1QBP (Revised Figure 1K).

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