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**ZFP91 disturbs metabolic fitness and antitumor activity of tumor-infiltrating T cells**

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Abstract

Proper metabolic activities facilitate T cell expansion and antitumor function; however, the mechanisms underlying disruption of the T cell metabolic programme and function in the tumor microenvironment (TME) remain elusive. Here, we show a Zinc finger protein 91 (ZFP91)-governed mechanism disrupting the metabolic pathway and antitumor activity of tumor-infiltrating T cells. Single-cell RNA sequencing revealed that impairments in T cell proliferation and activation correlated with ZFP91 in tissue samples from colorectal cancer patients. T cell-specific deletion of Zfp91 led to enhanced T cell proliferation and potentiated T cell antitumor function. Loss of ZFP91 increased mammalian target of rapamycin complex 1 (mTORC1) activity to drive T cell glycolysis. Mechanistically, T cell antigen receptor (TCR)-dependent ZFP91 cytosolic translocation promoted protein phosphatase 2A (PP2A) complex assembly, thereby restricting mTORC1-mediated metabolic reprogramming. Our results demonstrate that ZFP91 perturbs T cell metabolic and functional states in the TME and suggest that targeting ZFP91 may improve the efficacy of cancer immunotherapy.
**Introduction**

Tumor-infiltrating T cells play a major role in shaping antitumor immune responses, but these cells often exhibit a progressive loss of effector function in tumors (1, 2). Metabolic activity is critical for cell growth and proliferation and intimately linked to T cell fate and function (3, 4). Quiescent T cells rely on oxidative phosphorylation (OXPHOS) as a primary source of energy, whereas activated T cells shift to glycolytic metabolism to ensure the generation of sufficient biomass and energy (4). Proper metabolic activities facilitate T cell expansion and effector function, and alterations in metabolic programmes result in T cell functional disorders (5-7). Metabolic fitness in T cells is usually modified by the tumor microenvironment (TME) leading to T cell dysfunction (8, 9); however, the associated mechanisms are incompletely understood.

Signaling pathways are responsible for T cell metabolic reprogramming and antitumor activity in the TME (10, 11). The serine/threonine-protein phosphatase 2A (PP2A) has been implicated in regulating the mammalian target of rapamycin (mTOR) signaling to sustain the metabolic programmes and effector function of T cells (12, 13). Elevated PP2A activity in regulatory T (Treg) cells maintains a metabolic profile essential for Treg cell suppressive function (12, 14). Potassium-mediated suppression of T cell function in tumors is dependent on PP2A phosphatase activity (13). Silencing *Ppp2r2d*, a regulatory subunit of PP2A, enhances the cytotoxic function of tumor-infiltrating lymphocytes (TILs) (15, 16). Pharmacological inhibition of PP2A synergizes with immune checkpoint blockade to strengthen antitumor immune
responses in the treatment of solid tumors (17). These findings suggest that PP2A acts as a negative regulator of antitumor T cell immunity. It is well known that PP2A is the assembly product of three distinct subunits, the structural A (PP2Aα), regulatory B (PP2Ab), and catalytic C (PP2Ac) subunits (18). Nevertheless, the underlying mechanisms that orchestrate the holoenzyme assembly of PP2A to disrupt the T cell metabolic pathway and antitumor functions remain unclear.

Zinc finger protein 91 (ZFP91) as an E3 ubiquitin ligase is a potentially important oncogenic protein in multiple tumor types and its oncogenic activities include suppressing tumor apoptosis, promoting tumor growth, and accelerating tumor metastasis (19-21). The physiological function of ZFP91 in effector T cell activity in the TME is still unclarified. In this study, our single-cell RNA sequencing (scRNA-seq) analysis of tumor-infiltrating T cells revealed that impairments in T cell proliferation and activation correlated with ZFP91 in tissue samples from colorectal cancer (CRC) patients. Using a mouse strain with a conditional targeted deletion of Zfp91 in T cells, we found that ZFP91 dampened T cell antitumor function in vivo and inhibited T cell activation and proliferation in vitro. In consequence, targeting ZFP91 in T cells synergized with immunotherapy to delay tumor growth. Mechanistically, T cell antigen receptor (TCR)-induced ZFP91 cytosolic translocation facilitated ZFP91-mediated PP2Ac ubiquitination and PP2A holoenzyme assembly, thereby inhibiting mTORC1-mediated T cell glycolytic metabolism and antitumor function. Our results demonstrate that ZFP91 induces PP2A complex assembly to repress T cell metabolic and functional fitness in the TME. These findings suggest that targeting
ZFP91 holds great promise to unleash the full antitumor activity of tumor-infiltrating T cells and could help in the design of innovative strategies to improve the efficacy of cancer immunotherapy.
Results

Impairments in T cell proliferation and activation correlate with ZFP91 in CRC

To investigate the potential function of ZFP91 in T cell activity in the TME, we analyzed the mRNA expression profile of ZFP91 in different cell types from colon adenocarcinoma (COAD) tissue using the scRNA-seq database in Tumor Immune Single-cell Hub (TISCH) (22-26). Compared with other cell types, proliferating Mki67+ T cells exhibited relatively high mRNA expression levels of ZFP91 in seven scRNA-seq human and mouse COAD datasets (Figure 1A), indicating that ZFP91 may participate in the regulation of T cell proliferation in the TME. To further explore the role of ZFP91 in tumor-infiltrating T cells, we analyzed the correlation between T cell proliferation and ZFP91 mRNA expression in The Cancer Genome Atlas (TCGA) COAD dataset (27). Interestingly, a significantly positive correlation between the Gene Set Variation Analysis (GSVA) score of negative regulation of T cell proliferation and ZFP91 expression was observed in patients with colorectal cancer (CRC) (Figure 1B). Using previously published scRNA-seq data for human colorectal TILs (22), we found that ZFP91-silenced CD8+ T cells exhibited enrichment in T cell proliferation-related genes (Figure 1C). Furthermore, the expression of T cell activation-associated genes was also upregulated in ZFP91-silenced T cells from CRC tissue (Figure 1D). Subsequently, we verified these findings using CRC tissue samples. Indeed, tumor-infiltrating T cells with low ZFP91 expression contained abundant transcription of genes associated with T cell proliferation and activation.
These data suggest that impairments in T cell proliferation and activation correlate with ZFP91 in CRC.

**ZFP91 dampens T cell antitumor functions**

To verify the physiological importance of ZFP91 in T cell antitumor activity, we crossed Zfp91-flox mice with Cd4-Cre mice to generate Zfp91 T cell-conditional knockout mice (Zfp91fl/flCd4-Cre), in which ZFP91 was specifically deleted in T cells (Supplementary Figure 1A). T cell-specific deletion of Zfp91 did not affect T cell development or peripheral T cell homeostasis in 6-week-old mice (Supplementary Figure 1, B and C). In addition, the Treg cell percentages in the thymus, spleen and peripheral lymph nodes of 6-week-old Zfp91+/+Cd4-Cre mice and Zfp91fl/flCd4-Cre mice were comparable (Supplementary Figure 1D). Using an MC38 COAD model, we examined the role of ZFP91 in regulating antitumor immune responses. Compared to wild-type mice, Zfp91fl/flCd4-Cre mice had profound reductions in MC38 tumor size and tumor-induced lethality (Figure 2, A and B). In addition, MC38 cell-challenged Zfp91fl/flCd4-Cre mice had an increased frequency of interferon gamma (IFN-γ)–producing CD4+ and CD8+ effector T cells infiltrating the tumors (Figure 2, C and D). Parallel studies revealed that the Zfp91fl/flCd4-Cre mice also displayed enhanced antitumor immunity in a B16 melanoma model (Supplementary Figure 2, A-C).

To confirm the role of ZFP91 in T cell antitumor function, we adoptively transferred CD4+ or CD8+ T cells isolated from Zfp91fl/flCd4-Cre ovalbumin (OVA)-specific TCR-transgenic (OT-II or OT-I, respectively) mice into MC38-OVA
tumor-bearing wild-type mice. As expected, the mice transferred with
Zfp91\textsuperscript{fl/fl}Cd4-Cre OT-II T cells displayed more suppressed tumor growth and
prolonged survival (Figure 2, E and F). Consistently, the mice receiving
Zfp91\textsuperscript{fl/fl}Cd4-Cre OT-I T cells exhibited much greater efficiency in controlling tumor
growth and tumor-induced lethality (Figure 2, G and H). Collectively, these results
suggest that ZFP91 in T cells perturbs antitumor immune responses.

ZFP91 disturbs antitumor activity of tumor-infiltrating T cells

To demonstrate that ZFP91 disturbs T cell antitumor activity in the TME, we isolated
tumor-infiltrating T cells from tumor-bearing Zfp91\textsuperscript{+/+}Cd4-Cre mice and
Zfp91\textsuperscript{fl/fl}Cd4-Cre mice for the transcriptomic analysis. We noticed that negative
regulation of immune effector process was downregulated in both CD8\textsuperscript{+} and CD4\textsuperscript{+} T
cells from the tumors of Zfp91\textsuperscript{fl/fl}Cd4-Cre mice (Figure 3, A and B). By contrast,
positive regulation of adaptive immune response was upregulated in tumor-infiltrating
ZFP91-deficient CD8\textsuperscript{+} T cells (Figure 3A). Gene set enrichment analysis (GSEA) also
confirmed that effector T cell signatures were enriched in tumor-infiltrating
ZFP91-deficient CD8\textsuperscript{+} T cells (Figure 3C). To better define the phenotypes of
tumor-infiltrating ZFP91-deficient T cells, we adoptively transferred CFSE-labeled
WT OT-I cells and CTV-labeled ZFP91-deficient OT-I cells to the same
tumor-bearing mice. Notably, increased intratumoral infiltration of ZFP91-deficient
CD8\textsuperscript{+} T cells was observed in the tumor-bearing mice with transferred WT and
ZFP91-deficient OT-I cells (Figure 3D). Importantly, IFN-\(\gamma\)-producing
ZFP91-deficient OT-I cells were dramatically accumulated in the TME, although the
frequency of IFN-γ-producing WT OT-I cells and ZFP91-deficient OT-I cells was similar (Figure 3, E and F). Moreover, ZFP91-deficient OT-I cells expressed higher levels of Ki-67 compared to WT OT-I cells in the TME (Figure 3G). By contrast, splenic WT OT-I cells and ZFP91-deficient OT-I cells displayed comparable levels of IFN-γ and Ki-67 in the tumor-bearing mice with transferred WT and ZFP91-deficient OT-I cells (Supplementary Figure 3, A-D). These data suggest that ZFP91 is indispensable for the functional states of tumor-infiltrating T cells.

**ZFP91 inhibits T cell activation and proliferation**

To understand the mechanism by which ZFP91 regulates T cell activity, we performed transcriptomic analysis of Zfp91+/Cd4-Cre and Zfp91+/Cd4-Cre CD90.2+ T cells stimulated with anti-CD3 and anti-CD28 antibodies for 24 hours (Figure 4A). Interestingly, the levels of genes involved in T cell activation and proliferation were upregulated in ZFP91-deficient T cells (Figure 4, B and C). Moreover, ZFP91 deficiency augmented the TCR and CD28-stimulated production of effector cytokines, such as IFN-γ and interleukin-2 (IL-2), in ZFP91-deficient CD8+ T cells (Figure 4D). In addition, the expression of the cell proliferation marker Ki-67 observed following TCR and CD28 stimulation was increased in ZFP91-deficient CD4+ T cells and CD8+ T cells (Figure 4E). Consistently, enhanced proliferation of ZFP91-deficient CD4+ T cells and CD8+ T cells was observed after TCR and CD28 stimulation (Figure 4F). However, ZFP91-deficient and wild-type CD4+ T cells were similar in T cell differentiation (Supplementary Figure 4, A and B), suggesting that ZFP91 is
dispensable for T cell differentiation. These results indicate crucial roles for ZFP91 in restraining T cell activation and proliferation.

**ZFP91 suppresses T cell glycolytic metabolism**

Glucose metabolism is required for T cell activation, proliferation, and effector functions (4). Our transcriptomic analysis revealed that the levels of genes associated with glycolysis, including *Slc2a1, Slc2a3, Hk3*, and *Aldoc*, were upregulated in ZFP91-deficient T cells (Figure 5A), which prompted us to examine the involvement of ZFP91 in T cell metabolic programmes. Indeed, ZFP91-deficient T cells had significantly higher baseline and maximum glycolytic rates than did ZFP91-sufficient T cells after TCR and CD28 stimulation (Figure 5B), indicating that ZFP91 regulates T cell glycolysis. In contrast, stimulated ZFP91-deficient and ZFP91-sufficient T cells displayed similar OXPHOS rates, both at baseline and at maximum capacity (Figure 5C), suggesting a dispensable role for ZFP91 in regulating T cell OXPHOS. Increased aerobic glycolysis supports rapid T cell growth (28). In line with this, ZFP91-deficient T cells exhibited increases in cell size (Figure 5D). Consistently, high glucose transporter 1 (Glut1) surface expression, which is associated with increased glycolytic activity, was detectable in tumor-infiltrating ZFP91-deficient T cells (Figure 5E). Moreover, tumor-infiltrating ZFP91-deficient OT-I cells had higher levels of Glut1 compared to WT OT-I cells in the tumor-bearing mice with transferred WT and ZFP91-deficient OT-I cells (Figure 5F). By contrast, splenic WT OT-I cells and ZFP91-deficient OT-I cells displayed comparable levels of Glut1 in the tumor-bearing mice with transferred OT-I cells (Figure 5F). To define the dependence of effector
cytokine production and Ki-67 upregulation on glucose availability, we further activated T cells in the presence or absence of a glycolysis inhibitor, 2-deoxy-D-glucose (2-DG). After the addition of 2-DG, effector cytokine production and Ki-67 expression were greatly diminished in either wild-type T cells or ZFP91-deficient T cells (Figure 5, G and H). Importantly, stimulated ZFP91-deficient and ZFP91-sufficient T cells displayed similar levels of effector cytokine production and Ki-67 expression after 2-DG treatment (Figure 5, G and H). Therefore, ZFP91 restricts glycolytic metabolism to restrain T cell activity.

**ZFP91 inhibits mTORC1-dependent metabolic reprogramming**

Given that mTOR signaling is involved in metabolic programmes to modulate T cell activity (10), we sought to determine whether ZFP91 regulates the activity of the mTOR signaling pathway. Interestingly, immunoblot (IB) and flow cytometry analysis showed increased phosphorylation of ribosomal protein S6, ribosomal protein S6 kinase 1 (S6K1) and translation-initiation inhibitor 4EBP1 in ZFP91-deficient T cells after TCR and CD28 stimulation (Figure 6, A-C), suggesting that ZFP91 inhibits mTORC1 activation. However, mTORC2 activity (AKT phosphorylation at Ser473) in ZFP91-sufficient T cells was unaltered (Figure 6A). To determine the contribution of elevated mTORC1 to ZFP91-deficient T cell activity, we treated ZFP91-sufficient T cells with rapamycin to lower mTORC1. Rapamycin treatment reduced the extracellular acidification rate and erased the differences between ZFP91-deficient and ZFP91-sufficient T cells (Figure 6D). Rapamycin diminished effector cytokine production and Ki-67 expression, and rapamycin-treated ZFP91-deficient and
ZFP91-sufficient T cells showed comparable levels of effector cytokine production and Ki-67 expression (Figure 6, E and F). We further generate Zfp91^{fl/fl}Raptor^{fl/fl}Cd4-Cre mice to confirm the role of elevated mTORC1 to ZFP91-deficient T cell activity. Compared with stimulated Zfp91^{fl/fl}Cd4-Cre T cells, which showed increased glycolytic rates, stimulated T cells from Zfp91^{fl/fl}Raptor^{fl/fl}Cd4-Cre mice displayed markedly decreased glycolytic rates (Figure 6G). Importantly, Raptor deficiency eliminated the differences in glycolytic rates between ZFP91-deficient T cells and ZFP91-sufficient T cells (Figure 6G). Consistent with that finding, stimulated Zfp91^{fl/fl}Raptor^{fl/fl}Cd4-Cre T cells had less effector cytokine production and lower Ki-67 expression than did stimulated Zfp91^{fl/fl}Cd4-Cre T cells (Figure 6, H and I). These results indicate that ZFP91 restrains T cell glycolysis in an mTORC1-dependent manner.

**ZFP91 sustains PP2A activity in T cells**

PP2A is involved in the regulation of mTORC1 activity in T cells (12, 13). Mass spectrometry analysis has indicated an interaction between ZFP91 and PP2Ac (29). When we co-expressed ZFP91 and PP2Ac in HEK293T cells, the ZFP91-PP2Ac interaction was detectable (Supplementary Figure 5A). In addition, the endogenous ZFP91-PP2Ac association was observed in stimulated T cells (Supplementary Figure 5B). To examine whether ZFP91 regulates PP2A activity in T cells, we treated T cells with sphingomyelinase (SMase), which induces ceramide production (12), to activate PP2A phosphatase activity. Treatment of wild-type T cells with SMase significantly diminished the phosphorylation of S6 (Figure 7A). Moreover, SMase treatment
lowered the extracellular acidification rate (ECAR) in wild-type T cells (Figure 7B).
SMase also reduced the levels of cell size and Ki-67 expression of stimulated
wild-type T cells (Figure 7 C and D). However, SMase did not alter S6
phosphorylation levels, glycolytic rates, cell size, or Ki-67 expression in stimulated
ZFP91-deficient T cells (Figure 7, A-D). Furthermore, pharmacological inhibition of
PP2A with LB-100 increased S6 phosphorylation, the ECAR, cell size and Ki-67
expression in stimulated wild-type T cells (Figure 7, E-H). By contrast,
ZFP91-deficient T cells treated with or without LB-100 displayed no apparent
differences in S6 phosphorylation, maximum glycolytic rates, cell size or Ki-67
expression (Figure 7, E-H). Therefore, ZFP91 is indispensable for PP2A activity in T
cells.

To confirm the functional importance of ZFP91 in T cell PP2A activity, we
transduced activated wild-type and ZFP91-deficient T cells with or without PP2Ac
(Figure 7I). PP2Ac-overexpression in wild-type T cells led to the inhibition of
mTORC1 activity, coupled with lower levels of glycolytic rates, cell size, and Ki-67
expression (Figure 7, J-M). However, PP2Ac overexpression in ZFP91-deficient T
cells did not affect the abundance of phosphorylated S6, glycolytic rates, cell size, and
Ki-67 expression (Figure 7, J-M). Therefore, ZFP91 is indispensable for PP2A
function to inhibit mTORC1-dependent glycolysis in T cells.

**ZFP91 is required for PP2A complex assembly in T cells**

ZFP91 acts as an E3 ubiquitin ligase and promotes protein ubiquitination (19). When
we co-expressed ZFP91 and PP2Ac with HA-tagged ubiquitin (Ub) in HEK293T cells,
we detected ubiquitination of PP2Ac (Figure 8A). However, ZFP91-triggered ubiquitination of PP2Ac was not detectable in the presence of K48-Ub (Figure 8A). However, overexpression of ZFP91 with K63-Ub but not K63R-Ub induced strong ubiquitination of PP2Ac (Figure 8A). Moreover, ZFP91 deficiency reduced the extent of the K63-linked ubiquitination of PP2Ac in activated T cells (Figure 8B), indicating that ZFP91 mediates PP2Ac K63-linked ubiquitination. Since K63-linked ubiquitin chain formation is a signal for protein-protein interactions (30), we next determined the role of ZFP91 in PP2A complex assembly. Overexpression of ZFP91 enhanced the interaction between PP2Aa and PP2Ac in HEK293T cells (Figure 8C). Consistently, the amount of PP2Aa-associated PP2Ac protein was dramatically decreased in stimulated ZFP91-deficient T cells (Figure 8D). Thus, ZFP91 in T cells mediates PP2Ac K63-linked ubiquitination to facilitate PP2A complex assembly. Notably, our co-immunoprecipitation (Co-IP) assay showed that the interaction between ZFP91 and PP2Ac was strengthened after TCR stimulation (Figure 8E). Furthermore, ZFP91 was predominantly localized in the nuclear fraction in resting T cells (Figure 8F). Upon TCR stimulation, ZFP91 rapidly translocated from the nucleus to the cytoplasm (Figure 8, F and G). A Co-IP assay using cytosolic fractionation revealed that the ZFP91-PP2Ac interaction occurred in the cytoplasm and that the TCR-induced cytosolic translocation of ZFP91 promoted ZFP91-PP2Ac association (Figure 8H). These data suggest that TCR-triggered ZFP91 cytosolic translocation promotes PP2Ac ubiquitination and PP2A complex formation to sustain PP2A phosphatase activity.
We next interrogated the expression profile of ZFP91 in conventional T cells in different conditions. Naive T cells expressed a lower level of ZFP91 protein, but T cell receptor engagement greatly induced ZFP91 expression (Figure 8I). To detect the induction of ZFP91 in tumor-infiltrating T cells, we injected OT-I CD8+ T cells into the tumor-bearing mice inoculated with both MC38 and MC38-OVA cancer cells. Compared with OT-I CD8+ T cells from MC38 tumors, OT-I CD8+ T cells from MC38-OVA tumors had higher level of ZFP91 (Figure 8J), suggesting a tumor antigen-specific induction of ZFP91 in tumor-infiltrating T cells. Given that ZFP91 is induced in TME and that ZFP91 suppresses T cell antitumor function, we hypothesized that targeting ZFP91 in T cells could improve antitumor immune response to checkpoint blockade. As expected, the combination of anti–PD-1 treatment and ZFP91 deletion in T cells efficiently inhibited tumor progression, as shown by the size of MC38 tumors and tumor-induced lethality (Figure 8, K and L). These results suggest that targeting ZFP91 in T cells may be an approach for promoting antitumor responses and improving tumor immunotherapy.
Discussion

The data presented here have demonstrated that ZFP91-dependent PP2A complex assembly disrupts the T cell metabolic pathway and effector function. ZFP91 deficiency impaired PP2A activity and resulted in excessive mTORC1 activity and hyperglycolysis in T cells, thereby, potentiating T cell activation and proliferation and boosting antitumor T cell immune responses. In response to TCR stimulation, ZFP91 rapidly translocated from the nucleus to the cytoplasm, which promoted ZFP91-PP2Ac interaction and PP2A complex formation in the cytoplasm. Importantly, impairments in T cell activation and proliferation correlated with ZFP91 in CRC. These results identified a ZFP91-governed mechanism disturbing the T cell metabolic programme and antitumor function in the tumor microenvironment.

T cell functional impairment in the tumor microenvironment is a hallmark of many cancers (31, 32). T cell dysfunction is generally associated with disrupted metabolic pathways, impaired effector functions, and multiple overexpressed inhibitory receptors (9, 31). Although checkpoint blockade immunotherapies targeting the inhibitory receptors have yielded clinical benefits, only a fraction of patients achieve sustained clinical responses (33). The molecular mechanisms controlling T cell functional defects in the tumor microenvironment need to be further determined. Using previously published scRNA sequencing data (22), we observed that the impairments in T cell activation and proliferation correlated with ZFP91 in CRC tissue samples. Correspondingly, T cell-specific deletion of Zfp91 led to enhanced T cell proliferation and potentiated T cell antitumor function in mouse cancer models.
Our transcriptomic analysis revealed that loss of ZFP91 augmented the expression of genes involved in T cell activation, proliferation and glycolysis. In activated T cells, ZFP91 deficiency increased mTORC1 activity to increase glycolytic activity, thereby supporting T cell expansion and antitumor functions. These results identify ZFP91 as a metabolic regulator that perturbs T cell metabolic and functional states in the tumor microenvironment.

PP2A is the assembly product of the PP2Aα, PP2Aβ, and PP2Aγ subunits and regulates T cell metabolism and effector function (13, 14, 34, 35); however, how TCR stimulation directs the holoenzyme assembly of PP2A remains unclear. Our pharmacological experiments showed that ZFP91 was required for PP2A phosphatase activity to restrict mTORC1-mediated T cell glycolysis and proliferation. ZFP91 triggered PP2Aγ K63-linked ubiquitination, which provided a signal for PP2A complex assembly. Notably, ZFP91 was predominantly localized in the nucleus in resting T cells. Upon TCR stimulation, ZFP91 rapidly translocated from the nucleus to the cytoplasm and then promoted ZFP91-PP2Aγ association and PP2A complex formation in the cytoplasm. On the basis of these data, we propose that TCR-induced ZFP91 cytosolic translocation initiates PP2A holoenzyme assembly to perturb T cell metabolic and functional fitness.

ZFP91 is a potential oncogenic protein expressed in various tumor types and is required for tumorigenesis or tumor metastasis (20, 21). We found that ZFP91 in T cells diminished T cell proliferation and dampened antitumor T cell immune responses. Together with previous reports, our current study suggests that targeting
ZFP91 may not only suppress tumorigenesis or tumor metastasis but also boost antitumor T cell responses, and thus have important clinical applications. Indeed, it is still unclear how TCR signal triggers the cytosolic translocation of ZFP91, as well as the induction of ZFP91. Therefore, the upstream signals that control the induction and cytosolic translocation of ZFP91 in T cells remain to be further studied.

In summary, our findings identify a ZFP91-dependent mechanism sustaining the holoenzyme assembly of PP2A to restrict T cell metabolic and functional fitness. Our study not only defines ZFP91-mediated T cell glycolytic and functional defects in mouse tumor models but also highlights the correlation between T cell dysfunction and ZFP91 in CRC. Our results also suggest that targeting ZFP91 provides a promising strategy for the improvement of cancer immunotherapy.
**Methods**

**Human colorectal cancer samples.** Human colorectal cancer tissues were obtained at Ruijin Hospital, Shanghai, China. Solid cancer tissues were freshly isolated and digested. Tumor-infiltrating lymphocytes were enriched by density-gradient centrifugation. T cells were purified using the REAlease CD4/8 MicroBead Kit (130-121-561, Miltenyi).

**Mice.** Zfp91-floxed mice (in C57BL/6 background) were generated using a LoxP targeting system at the Shanghai Model Organisms Center, Inc. (Shanghai, China). The Zfp91-floxed mice were crossed with Cd4-Cre transgenic mice (The Jackson Laboratory) in B6 background to obtain age-matched Zfp91+/+ Cd4-Cre and Zfp91fl/fl Cd4-Cre mice for experiments. B6.SJL mice, OT-I TCR-transgenic mice and OT-II TCR-transgenic mice in C57BL/6 background were purchased from the Jackson Laboratory. The OT-I mice and OT-II mice were crossed with Zfp91fl/fl Cd4-Cre mice to produce Zfp91fl/fl Cd4-Cre OT-I mice and Zfp91fl/fl Cd4-Cre OT-II mice. The Raptor-floxed mice (in C57BL/6 background) were purchased from the Jackson Laboratory and crossed with Zfp91fl/fl Cd4-Cre mice to obtain Zfp91fl/fl Raptorfl/fl Cd4-Cre. Mice were randomly allocated to each experimental group. All mice used in experiments were age- and sex-matched. All mice maintained in pathogen-free facilities at the Institutional Animal care and Use Committee (IACUC) of Shanghai Jiao Tong University School of Medicine.

**Plasmids, antibodies, and reagents.** Mouse ZFP91, PP2Aa and PP2Ac genes were amplified from the mouse tissue cDNA library and subsequently cloned into lentivirus
vector pLVX-IRES-ZsGreen1. HA-tagged K48, K63 and K63R mutant ubiquitin were cloned into the pcDNA3-HA vector. Antibodies for p-S6 (4858s; 1:1000 for WB), PP2Aa (2041s; 1:1000 for WB), PP2Ac (2038s; 1:1000 for WB), p-Akt (S473) (4060s; 1:1000 for WB), p-4EBP1(Ser65) (9451s; 1:1000 for WB), p-p70 S6 Kinase (Thr389) (9234s; 1:1000 for WB) and K63 polyubiquitin (D7A11; 1:1000 for WB) were purchased from Cell Signaling Technology, Inc. HRP-conjugated anti-HA antibody (3F10; 1:2000 for WB) was from Roche, Inc. Anti-Flag (M2, F3165; 1:2000 for WB) antibody and β-actin (A2228-100UL; 1:5000 for WB) were from Sigma-Aldrich, Inc. Anti-His antibody (D291-3; 1:1000 for WB) was from Medical & Biological Laboratories. Anti-ZFP91 antibody (A303-245A; 1:1000 for WB) were purchased from Bethyl Laboratories. Anti-Lamin B antibody (66095-1-Ig; 1:1000 for WB) was from Proteintech. For flow cytometry experiments, antibodies for CD4 (GK1.5), CD8 (53-6.7), CD44 (IM7), CD62L (MEL-14), Ki-67 (So1A15), IFN-γ (XMG1.2), IL-17 (eBio17B7), Foxp3 (FJK-16s) were purchased from Thermo Fisher Scientific, Inc. Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP® Rabbit mAb (APC Conjugate) were purchased from Cell Signaling Technology, Inc. Anti-Glut1 was purchased from Abcam (ab115730). Fluorescence staining secondary antibody IFKine Red Donkey Anti-Mouse IgG was purchased from Abbkine (A24411-1). Anti-mouse-PD-1 (clone J43) and isotype control IgG (hamster IgG) antibodies were from Bio X Cell. Sphingomyelinase (SMase) from Staphylococcus aureus (S8633) was purchased from Sigma-Aldrich. LB-100 (S7537) was purchased from Selleck.
**Flow cytometry.** Freshly isolated cells were resuspended in PBS containing 2% fetal bovine serum (FBS) with indicated fluorochrome-conjugated antibodies for surface staining. Tumor-infiltrating lymphocytes were enriched by density-gradient centrifugation and stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin in the presence of monensin for 5 hours. After stimulation, cells were stained with anti-CD8 or anti-CD4 first, then fixed, permeabilized and stained with indicated antibodies for intracellular cytokine expression analysis. Stimulated T cells were treated with Fix Buffer I at 37°C for 10 min, incubated in pre-cold Perm Buffer III for another 20 min, and then stained with p-S6 antibody for flow cytometry analysis. For cell proliferation analysis, isolated T cells were labeled with 5μM CFSE, cultured with irradiated splenocytes depleted of T cells and stimulated with plate-bound anti-CD3 (1μg/ml) and anti-CD28 (1μg/ml) for 72 h. For Ki-67 detection, stimulated T cells were fixed, permeabilized and stained with indicated antibodies according to the manufacturer’s protocol (Thermo Fisher Scientific).

**T cell purification and cultures.** Peripheral CD4⁺, CD8⁺ or CD90.2⁺ T cells were isolated from mouse spleen and lymph node by positive selection magnetic beads (Miltenyi). These cells were stimulated with plate-bound anti-CD3 (1μg/ml) and anti-CD28 (1μg/ml), cultured with complete RPMI 1640 medium containing 10% FBS and antibiotics. 2×10⁵ T cells per well in 96-well plate were stimulated for flow cytometric analysis. 5×10⁶ T cells per well in 6-well plate were stimulated for Seahorse, RNA sequence, immunoblot and immunoprecipitation analysis.
Tumor infiltrating lymphocyte isolation. Tumors from tumor-bearing mice were collected, cut into 1-3 mm size pieces and digested for 30 min in RPMI 1640 medium containing 10% FBS, 0.2mg/mL Collagenase IV and 20μg/mL DNase I. Digested tissues were filtered through 100μm cell strainer and washed with PBS. 4 mL 40% Percoll was added to resuspend tissues and 2.5 mL 70% Percoll was added slowly from the bottom of tube. Centrifuge at 2500rpm for 20 min, acceleration 1 and deceleration 1. Lymphocytes were enriched at the bilayer interface and washed with PBS to obtain tumor infiltrating lymphocytes.

ELISA and quantitative RT-PCR. Supernatants of in vitro cell cultures were analyzed by ELISA using a commercial assay system (Thermo Fisher Scientific). Total RNA was extracted from isolated human tumor-infiltrating T cells and subjected to qRT-PCR using gene-specific primers. Data were generated with the comparative threshold cycle (Delta CT) method by normalizing to a reference gene, b-Actin. Sequences of primers used in the studies are listed as following. Zfp91-F:

GCCATCGTGATACAGAGAACACC, Zfp91-R:

CTAATGCCACCTGGAGACTGATG. Mki67-F:

GAAAGAGTGGCAACCTGCCTTC, Mki67-R:

GCCACCAAGTTTTACTACATCTGCC. Ptg1-F:

GCTTTGGGAACCTGTCACAGAGC, Ptg1-R:

CTGGATAGGCATCATCTGAGGC. Ccnb1-F:

GACCTGTGCAGGCTTTCTCTG, Ccnb1-R:

GGTATTTTGGTGACTGCTTGC. Tyms-F:
GGTGTGTTTGGAGGAGTTGCTGTG, Tyns-R:
GGAGAATCCCAGGCTGTCCAAA. Tbx21-F:
ATTGCCGTGACTGCTACCAGA, Tbx21-R:
GGAATTGACAGTTGGGTCCAGG. Gzma-F:
CCACACGCAGGTGACCTTAA, Gzma-R:
CCTGCAACTTGGCACATGGTTC. Ifng-F:
GAGTGTGAGACCATCAAGGAAG, Ifng-R:
TGCTTTGCGTTGGACATTCAGTC. Prf1 -F:
ACTCAGAGGCAGGGAATTTG, Prf1-R:
CTCTTGAGTACGGGTCAGCG. b-Actin-F:
CACCATTGGCAATGAGCGGTTC, b-Actin-R:
AGGTCTTGGATGTTCCACGT.

**Tumor model.** MC38 murine colon cancer cells (Kerafast), MC38-OVA murine colon cancer cells (Dr. Qiang Zou’s Lab) and B16-F10 murine melanoma cells (ATCC) were all tested negative for mycoplasma contamination. All the cell lines used in this study have been authenticated before. MC38 murine colon cancer cells and MC38-OVA murine colon cancer cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) medium supplemented with 10% FBS. B16-F10 murine melanoma cells were cultured with complete RPMI-1640 medium containing 10% FBS. These tumor cells (5×10^5 cells) were subcutaneously injected into 6 to 8-week-old mice. Tumor size was calculated by length × width. Mice bearing a tumor with a size that was larger than 225 mm^2 were euthanized. Tumor size and survival
were recorded from day 7 after tumor injection. To detect the induction of ZFP91 in tumor-infiltrating T cells, MC38 (5×10⁵ cells) and MC38-OVA (5×10⁵ cells) cancer cells were subcutaneous injected into the left and right flanks of C57BL/6 mice. 2×10⁶ OT-I CD8⁺ T cells were intravenous injected into tumor bearing mice at day 7 after tumor cells injection. At day 14, tumor infiltrating lymphocytes from MC38 and MC38-OVA tumor tissues were collected for further analysis. For cotransfer experiments, Zfp91⁺/⁺Cd4-Cre OT-I and Zfp91fl/flCd4-Cre OT-I cells were stimulated with 250 ng/mL OVA257-264 peptides plus 10 ng/mL IL-2 for two days and then cultured in fresh medium containing 10 ng/mL IL-2 for the other two days. Zfp91⁺/⁺Cd4-Cre (WT) OT-I cells were labeled with CFSE (5 μM) and Zfp91fl/flCd4-Cre (KO) OT-I cells were labeled with CTV (5 μM). CFSE-labeled WT OT-I cells and CTV-labeled KO OT-I cells were mixed at a 1:1 ratio (5×10⁶ cells each) and adoptively transferred into MC38-OVA tumor bearing mice at day 7 after tumor injection. Tumor tissues and spleen were harvested for subsequent experiments at day 7 after OT-I cell transfer.

**Lentivirus transduction.** HEK293T cells were transfected with lentivirus expression vectors and packing vectors to produce lentivirus particles. CD90.2⁺ T cells from Zfp91⁺/⁺Cd4-Cre and Zfp91fl/flCd4-Cre mice were stimulated with anti-CD3 (1μg/ml) and anti-CD28 (1μg/ml) for 24 hours, then infected with lentiviruses in the fresh medium containing IL-2 (10ng/mL), β-mercaptoethanol (50μM) and polybrene (6μg/mL). Transduced cells were enriched based on the expression of GFP after 3 days.
**RNA-sequencing analysis.** Wild-type (WT) and ZFP91-deficient (KO) CD90.2+ T cells were isolated from the spleen of tumor-free Zfp91+/+ Cd4-Cre and Zfp91fl/fl Cd4-Cre mice or the MC38 tumors of tumor-bearing Zfp91+/+ Cd4-Cre and Zfp91fl/fl Cd4-Cre mice. Naïve CD90.2+ T cells were stimulated with 6-well-plate-bound anti-CD3 (1µg/ml) and anti-CD28 (1µg/ml) for 24 hours. Total RNA was extracted from tumor-infiltrating T cells or *in vitro* activated T cells. Isolated total RNA was subjected to RNA-sequencing using Illumina Nextseq500 (75bp paired end reads). The raw reads were aligned to the mouse reference genome (version mm10) using Tophat2 RNASeq alignment software. The mapping rate was about 96% overall across all the samples in the dataset. The raw reads were processed to generate read counts for every gene. The read counts were normalized using R package DESeq2, and then were centered and scaled for each gene, generating z-scores. P-values obtained from multiple tests were adjusted using Benjamini-Hochberg correction. The RNA-seq data reported in this paper are available under accession number PRJNA646554 (NCBI Trace and Short-Read Archive).

**Glycolytic and mitochondrial respiration rate measurement.** Peripheral CD4+, CD8+ or CD90.2+ T cells were stimulated with anti-CD3 and anti-CD28 for 24 hours and plated at 2×10⁵ cells per well in a 96-well Seahorse assay plate. ECAR and OCR measurements were conducted with the Seahorse XFe96 Extracellular Flux Analyzer (Aglient Technologies). ECAR (mpH/min) and OCR (pmol/min) were measured as indicated upon cell treatment with glucose (10mM), oligomycin (2µM), 2-DG.
(50mM), FCCP (1μM), rotenone/antimycin A (0.5μM) according to manufacturer’s instructions. The XF Cell Mito and the XF Glycolytic stress test kits were purchased from Agilent Technologies, Inc.

**Immunoblot and ubiquitination assays.** Stimulated T Cells were rinsed once with ice-cold PBS and lysed with RIPA buffer containing protease inhibitor on ice for 30 min. The cell lysates were clarified by centrifugation at 14000 r.p.m for 10 min. For immunoprecipitations, the supernatants were immunoprecipitated with indicated antibodies overnight. The protein G-agarose beads were washed three times with lysis buffer, then incubated with the supernatants for another 2 hours. After incubation, immunoprecipitated proteins were washed three times with lysis buffer. Samples were denatured by the addition of sample buffer and boiling for 10 min, resolved by 8-16% SDS-PAGE and analysed by immunoblotting. For ubiquitination assays, immunoprecipitated PP2Ac from stimulated T cells was detected by immunoblot using ubiquitin antibodies.

**Statistics.** Statistical analysis was performed using Prism software (GraphPad Prism version 8.0). Two-tailed unpaired Student’s t tests were performed and indicated in figure legends. Survival curves were analyzed by log-rank (Mantel–Cox) test. The data are presented as means ± SEM, and n is indicated in figure legends. P value <0.05 is considered statistically significant, and the level of significance is indicated as ns, not statistically significant; *P < 0.05 and **P < 0.01.

**Study approval.** The human colorectal cancer sample collection was approved by the Clinical Research Ethics Committee of Ruijin Hospital and complied with all relevant
ethical regulations (2019-205). All animal procedures were performed according to protocols approved by the IACUC at the Shanghai Jiao Tong University School of Medicine.
Author contributions

FW performed the experiments, analyzed the data, and wrote the paper. YZ, XY, XT, RD, ZH, and AW helped with mouse experiments and flow cytometry, and analyzed the data. ZW, YY, and QZ designed the experiments, interpreted the results, wrote the paper, and oversaw the research project.
Acknowledgments

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References


**Figure 1**

A) Heatmap showing mean expression across different datasets. The expression levels are color-coded, with purple indicating lower expression and red indicating higher expression.

B) Scatter plot from the TCGA-COAD database showing the negative regulation of T cell proliferation against Zfp91 expression. The line of best fit has a correlation coefficient of Rs = 0.35 and a p-value of 1.1x10^-9.

C) Enrichment score for CD8+ T cell proliferation with Zfp91 expression (NES = -1.6) and Zfp91 silence (NES = -1.3).

D) Enrichment score for T cell activation with Zfp91 expression (NES = -1.3) and Zfp91 silence (NES = -1.3).

E) Zfp91-high and Zfp91-low T cells are compared for gene expression levels in representative genes.

F) Zfp91-high and Zfp91-low T cells are compared for gene expression levels in representative genes.
Figure 1. Impairments in T cell proliferation and activation are correlated with ZFP91 in CRC. (A) The heatmap showing average mRNA expression of ZFP91 (top) and the violin plot showing the distribution of ZFP91 mRNA expression (bottom) in different cell-types from seven scRNA-seq datasets for COAD. B, B cells. CD4 Tconv, CD4^+ conventional T cells. CD8 T, CD8^+ T cells. CD8 Tex, exhausted CD8^+ T cells. Mast, mast cells. Mono/Macro, monocytes and macrophages. NK, natural killer cells. Mki67^+ T, proliferating Mki67^+ T cells. Treg, regulatory T cells. pDC, plasmacytoid dendritic cells. (B) The Spearman correlation of mRNA expression of ZFP91 and GSVA score of negative regulation of T cell proliferation in the TCGA COAD database. (C and D) GSEA of the signature genes of regulation of CD8 positive alpha beta T cell proliferation (C) and alpha beta T cell activation (D) in ZFP91 expressed and ZFP91 silenced T cells. NES, normalization enrichment score. (E and F) qRT-PCR analysis of genes associated with T cell proliferation (E) and activation (F) in tumor-infiltrating T cells from CRC. The normalized Zfp91 expression value of tumor-infiltrating T cells with the lowest expression of Zfp91 was set to be 1. The normalized Zfp91 expression values of Zfp91^{high} T cells were more than 2 (n=4) and those of Zfp91^{low} T cells were less than 2 (n=6). Representative data are shown from three (E and F) independent experiments. Data are represented as means ± SEM. *, P < 0.05; **, P < 0.01.
Figure 2
**Figure 2. ZFP91 dampens T cell antitumor functions.** (A and B) Tumor progression (A) and survival curves (B) of Zfp91<sup>+/+</sup>Cd4-Cre and Zfp91<sup>fl/fl</sup>Cd4-Cre mice given subcutaneous injection of 5×10<sup>5</sup> MC38 murine colon cancer cells (n=8).

(C and D) Flow cytometric analysis of T cells in tumors of Zfp91<sup>+/+</sup>Cd4-Cre and Zfp91<sup>fl/fl</sup>Cd4-Cre mice injected s.c. with MC38 murine colon cancer cells (day 14). The data are presented as representative plots (C) and as summary graphs (D) (n=5).

(E and F) Tumor progression (E) and survival curves (F) of B6.SJL mice given intravenous injection of 2×10<sup>6</sup> Zfp91<sup>+/+</sup>Cd4-Cre OT-II or Zfp91<sup>fl/fl</sup>Cd4-Cre OT-II CD4<sup>+</sup> T cells at day 7 after subcutaneous injection of 5×10<sup>5</sup> MC38-OVA colon cancer cells (n=6).

(G and H) Tumor progression (G) and survival curves (H) of B6.SJL mice given intravenous injection of 2×10<sup>6</sup> Zfp91<sup>+/+</sup>Cd4-Cre OT-I and Zfp91<sup>fl/fl</sup>Cd4-Cre OT-I CD8<sup>+</sup> T cells at day 7 after subcutaneous injection of 5×10<sup>5</sup> MC38-OVA colon cancer cells (n=7). Experiments were independently repeated three times. Data are presented as the mean ± SEM. *, P < 0.05; **, P < 0.01. (A), (D), (E), and (G) were analyzed by two-tailed Student’s t test, and (B), (F), and (H) were analyzed by log-rank (Mantel-Cox) test.
Figure 3
Figure 3. ZFP91 disturbs antitumor activity of tumor-infiltrating T cells. (A and B) Gene ontology enrichment analysis of upregulated (Up) and downregulated (Down) gene sets in tumor-infiltrating CD8+ T cells (A) or CD4+ T cells (B) of Zfp91+/+ Cd4-Cre and Zfp91fl/fl Cd4-Cre mice (n=6) injected s.c. with MC38 murine colon cancer cells (day 14). (C) GSEA enrichment plots of indicated signatures in tumor-infiltrating CD8+ T cells of Zfp91+/+ Cd4-Cre (WT) and Zfp91fl/fl Cd4-Cre (KO) mice (n=6) injected s.c. with MC38 murine colon cancer cells (day 14). (D) Number of OT-I cells in tumor tissues from MC38-OVA tumor bearing mice with transferred CFSE-labeled WT OT-I and CTV-labeled KO OT-I cells (at day 7 after OT-I cells adoptive transfer) (n=4). (E-G) Percentage and number of IFN-γ+ OT-I cells (E and F) or Ki-67+ OT-I cells (G) in tumor tissues from MC38-OVA tumor bearing mice with transferred OT-I cells (at day 7 after OT-I cells adoptive transfer) (n=4).

Experiments were independently repeated four times (D-G). Data are presented as the mean ± SEM. ns, not statistically significant; **, P < 0.01; two-tailed Student’s t test.
Figure 4
Figure 4. ZFP91 deficiency promotes T cell activation and proliferation. (A) Volcano plot comparing global gene-expression profiles between Zfp91<sup>+/+</sup>Cd4-Cre (WT) and Zfp91<sup>fl/fl</sup>Cd4-Cre (KO) CD90.2<sup>+</sup> T cells stimulated with anti-CD3 and anti-CD28 for 24 hours. The red dots represented the transcripts with increasing expression value while the blue dots signified transcripts with decreasing expression value in Zfp91<sup>fl/fl</sup>Cd4-Cre CD90.2<sup>+</sup> T cells. (B) Gene ontology enrichment analysis of upregulated (Up) and downregulated (Down) gene sets in Zfp91<sup>fl/fl</sup>Cd4-Cre (KO) T cells stimulated with anti-CD3 and anti-CD28 for 24 hours. (C) A heatmap of upregulated genes associated with T cell activation and proliferation in ZFP91-deficient T cells relative to those in wild-type T cells. (D) Enzyme-linked immunosorbent assay (ELISA) of IFN-γ and IL-2 in supernatants of Zfp91<sup>+/+</sup>Cd4-Cre and Zfp91<sup>fl/fl</sup>Cd4-Cre CD8<sup>+</sup> T cells stimulated with anti-CD3 and anti-CD28 (n=4). (E) Flow cytometry analysis of Ki-67 expression in Zfp91<sup>+/+</sup>Cd4-Cre and Zfp91<sup>fl/fl</sup>Cd4-Cre CD4<sup>+</sup> or CD8<sup>+</sup> T cells stimulated with anti-CD3 and anti-CD28 for 48 hours (n=3). (F) Zfp91<sup>+/+</sup>Cd4-Cre and Zfp91<sup>fl/fl</sup>Cd4-Cre CD4<sup>+</sup> or CD8<sup>+</sup> T cells (3×10<sup>5</sup> cells per well) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured with irradiated splenocytes depleted of T cells (1×10<sup>5</sup> cells per well) in the presence of anti-CD3 and anti-CD28 for 72 hours for T cell proliferation assay (n=3). Experiments were independently repeated three times. Data are presented as the mean ± SEM. *, P < 0.05; **, P < 0.01; two-tailed Student’s t test.
**Figure 5**
Figure 5. ZFP91 suppresses T cell glycolytic metabolism. (A) A heatmap of upregulated genes associated with T cell glycolysis in ZFP91-deficient T cells relative to those in wild-type T cells stimulated with anti-CD3 and anti-CD28 for 24 hours. (B) Extracellular acidification rate (ECAR) of Zfp91+/+Cd4-Cre and Zfp91fl/flCd4-Cre CD4+ or CD8+ T cells stimulated with anti-CD3 and anti-CD28 for 24 hours at base line and in response to glucose (Glu), oligomycin (Oli) and 2-DG (n=3 or 4). (C) Oxygen consumption rates (OCR) of Zfp91+/+Cd4-Cre and Zfp91fl/flCd4-Cre CD4+ or CD8+ T cells stimulated with anti-CD3 and anti-CD28 for 24 hours at base line and in response to oligomycin (Oligo), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and rotenone plus antimycin (R+A) (n=3 or 4). (D) Flow cytometric analysis of cell size (forward scatter area (FSC-A)) in Zfp91+/+Cd4-Cre and Zfp91fl/flCd4-Cre CD4+ or CD8+ T cells stimulated with anti-CD3 and anti-CD28 for 24 hours. (E) Flow cytometric analysis of Glut1 expression in T cells in tumors of Zfp91+/+Cd4-Cre and Zfp91fl/flCd4-Cre mice injected s.c. with MC38 murine colon cancer cells (day 14). (F) Number of Glut1+ OT-I cells in the spleen and tumor from MC38-OVA tumor bearing mice with transferred OT-I cells (at day 7 after OT-I cells adoptive transfer) (n=4). (G and H) ELISA assay of IFN-γ and IL-2 in supernatants (G, n=4) and flow cytometric analysis of Ki-67 expression (H, n=3) of Zfp91+/+Cd4-Cre (WT) and Zfp91fl/flCd4-Cre (KO) T cells stimulated with anti-CD3 and anti-CD28 for 48 hours in the presence of 2-DG (2mM). Experiments were independently repeated three times. Data are presented as the mean ± SEM. ns, not statistically significant; **, P < 0.01; two-tailed Student’s t test.
Figure 6
Figure 6. Regulation of T cell glycolysis by ZFP91 is dependent on mTORC1. (A) Immunoblot analysis of indicated proteins in Zfp91+/+ Cd4-Cre and Zfp91fl/fl Cd4-Cre T cells stimulated with anti-CD3 and anti-CD28. (B) Quantification of indicated protein: β-actin levels in Zfp91+/+ Cd4-Cre and Zfp91fl/fl Cd4-Cre T cells stimulated with anti-CD3 and anti-CD28 for 2 hours (n=3). (C) Flow cytometric analysis of p-S6 level in Zfp91+/+ Cd4-Cre and Zfp91fl/fl Cd4-Cre CD4+ or CD8+ T cells stimulated with anti-CD3 and anti-CD28 for 2 hours. (D-F) ECAR (D, n=4), ELISA assay of IFN-γ and IL-2 in supernatants (E, n=4), flow cytometric analysis of Ki-67 expression (F, n=3) of Zfp91+/+ Cd4-Cre (WT) and Zfp91fl/fl Cd4-Cre (KO) T cells stimulated with anti-CD3 and anti-CD28 for 24 hours (D) or 48 hours (E and F) in the presence of rapamycin (Rap, 1mM). (G-I) ECAR (G, n=4), ELISA assay of IFN-γ and IL-2 in supernatants (H, n=3), flow cytometric analysis of Ki-67 expression (I, n=3) of Zfp91+/+ Cd4-Cre, Zfp91fl/fl Cd4-Cre, Raptorfl/fl Cd4-Cre and Zfp91fl/fl Raptorfl/fl Cd4-Cre (DKO) CD90.2+ T cells stimulated with anti-CD3 and anti-CD28 for 24 hours (G) or 48 hours (H and I). Experiments were independently repeated three times. Data are presented as the mean ± SEM. ns, not statistically significant; *, P < 0.05; **, P < 0.01; two-tailed Student’s t test.
Figure 7
Figure 7. ZFP91 is required for PP2A activity in T cells. (A) Flow cytometric analysis of p-S6 level in Zfp91+/+Cd4-Cre (WT) and Zfp91fl/flCd4-Cre (KO) CD90.2+ T cells stimulated with anti-CD3 and anti-CD28 for 2 hours in the presence of SMase (0.5 U/ml) or Ctrl (50% Glycerol in PBS). (B) ECAR of WT and KO T cells stimulated with anti-CD3 and anti-CD28 for 24 hours in the presence of SMase (n=3). (C and D) Flow cytometric analysis of cell size (C) and Ki-67 expression (D, n=3) in WT and KO T cells stimulated with anti-CD3 and anti-CD28 for 24 hours (for FSC-A detection) or 48 hours (for Ki-67 detection) in the presence of SMase. (E) Flow cytometric analysis of p-S6 level in WT and KO T cells stimulated with anti-CD3 and anti-CD28 for 2 hours in the presence of LB-100 (1mM). (F) ECAR of WT and KO T cells stimulated with anti-CD3 and anti-CD28 for 24 hours in the presence of LB-100 (1mM). (G and H) Flow cytometric analysis of cell size (G) and Ki-67 expression (H, n=3) in WT and KO T cells stimulated with anti-CD3 and anti-CD28 for 24 hours (for FSC-A detection) or 48 hours (for Ki-67 detection) in the presence of LB-100. (I) IB analysis of HA-PP2Ac in WT and KO T cells transduced with either an empty vector (EV) or PP2Ac expression. (J-M) p-S6 expression (J), ECAR (K, n=4), cell size (L), and Ki-67 expression (M, n=3) of transduced T cells from (I) stimulated for 2 h (J), 24 h (K and L), or 48 h (M) with antibodies against CD3 and CD28. Experiments were independently repeated three times. Data are presented as the mean ± SEM. ns, not statistically significant; *, P < 0.05; **, P < 0.01; two-tailed Student’s t test.
Figure 8
Figure 8. ZFP91 enforces PP2A holoenzyme assembly. (A) Immunoblot analysis of ubiquitinated PP2Ac in HEK293T cells transfected with indicated vectors. (B) Immunoblot analysis of PP2Ac K63-linked ubiquitination in Zfp91^{+/+}Cd4-Cre (WT) and Zfp91^{fl/fl}Cd4-Cre (KO) CD90.2^+ T cells stimulated with anti-CD3 and anti-CD28 for 4 hours. (C) Lysates from HEK293T cells transfected with the indicated vectors were subjected to immunoprecipitation (IP). (D) Lysates from WT and KO CD90.2^+ T cells stimulated with anti-CD3 and anti-CD28 for 4 hours were subjected to IP. (E) Lysates from WT CD90.2^+ T cells stimulated with anti-CD3 and anti-CD28 were subjected to IP. (F and G) IB analysis of the indicated proteins in whole cell lysates (WL), cytoplasmic fractions (CF) and nuclear fractions (NF) of WT CD90.2^+ T cells stimulated with anti-CD3 and anti-CD28. (H) IB and IP assays using the cytoplasmic fractions (CF) of WT T cells stimulated with anti-CD3 and anti-CD28. (I) Flow cytometry analysis of ZFP91 level in T cells stimulated with anti-CD3 and anti-CD28 for 24 hours. Isotype control (shaded); US, unstimulated; ST, stimulated. (J) Flow cytometric analysis of ZFP91 expression in OT-I cells in tumors of WT mice given intravenous injection of 2×10^6 OT-I cells at day 7 after subcutaneous injection of MC38 (left side) and MC38-OVA (right side) cancer cells (n=5). (K and L) Tumor growth (K) and survival curves (L) of Zfp91^{+/+}Cd4-Cre and Zfp91^{fl/fl}Cd4-Cre mice injected s.c. with MC38 cancer cells (n = 7) followed by intraperitoneal injection with PD-1 antibody on days 7, 10, and 13. Ctrl, control antibodies. Data are representative of three independent experiments and presented as the mean ± SEM. *, P < 0.05; **,
P < 0.01. (J) and (K) were analyzed by two-tailed Student’s t test, and (L) was analyzed by log-rank (Mantel-Cox) test.