AMP-activated protein kinase alpha1 promotes tumor development via FOXP3 elevation in tumor-infiltrating Treg cells

Highlights
- AMPKα1 is upregulated in tumor-infiltrated Treg cells
- Deficiency of AMPKα1 in Tregs prevents tumor development and promotes antitumor immunity
- AMPKα1 regulates Foxp3 protein stability through E3 ligase CHIP

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AMP-activated protein kinase alpha1 promotes tumor development via FOXP3 elevation in tumor-infiltrating Treg cells

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SUMMARY

Overwhelming evidence indicates that infiltration of tumors by Treg cells with elevated levels of FOXP3 suppresses the host antitumor immune response. However, the molecular mechanisms that maintain high expression of FOXP3 in tumor-infiltrating Treg cells remain elusive. Here, we report that AMP-activated protein kinase alpha1 (AMPKα1) enables high FOXP3 expression in tumor-infiltrating Treg cells. Mice with Treg-specific AMPKα1 deletion showed delayed tumor progression and enhanced antitumor T cell immunity. Further experiments showed that AMPKα1 maintains the functional integrity of Treg cells and prevents interferon-γ production in tumor-infiltrating Treg cells. Mechanistically, AMPKα1 maintains the protein stability of FOXP3 in Treg cells by downregulating the expression of E3 ligase CHIP (STUB1). Our results suggest that AMPKα1 activation promotes tumor growth by maintaining FOXP3 stability in tumor-infiltrating Treg cells and that selective inhibition of AMPK in Treg cells might be an effective anti-tumor therapy.

INTRODUCTION

The recognition of tumor antigens by the host immune system promotes antitumor immune responses (Carey et al., 1976). The promotion of tumor-specific T cell responses has been recognized as a promising strategy for cancer therapy (Borst et al., 2018). However, the increased expression of immunosuppressive molecules (PD1/PDL1) and enrichment of immunosuppressive cells (myeloid-derived suppressor cells, tumor-associated macrophages, and regulatory T (Treg) cells) in the tumor microenvironment (TME) limit antitumor immunity and promote tumor immune escape (Han et al., 2020; Kumar et al., 2016; Tanaka and Sakaguchi, 2017; Petty and Yang, 2017). Despite the great progress achieved with immune checkpoint blockers in cancer therapy, the molecular mechanisms that determine the immunosuppressive character of the TME are still not fully understood.

Treg cells are a heterogeneous population of lymphocytes that express the transcription factor Foxp3 and play an essential role in immune balance and tissue homeostasis (Smigliel et al., 2014; Veiga-Parga et al., 2013; Bonney et al., 2015). Stable expression of Foxp3 is required for the development and function of Treg cells, whereas deficiency or mutation of Foxp3 impairs Treg function and causes several autoimmune disorders (Fontenot et al., 2003; Bennett et al., 2001; Bacchetta et al., 2018). In cancers, enrichment of Treg cells in the TME promotes tumor development, invasiveness, and metastasis (Hatzioannou et al., 2017), whereas depletion or inhibition of Treg cells in the TME has found to be a promising strategy for cancer therapy (Xiong et al., 2020; Hatzioannou et al., 2020). In contrast to Treg cells in peripheral blood and other tissues, Treg cells that infiltrate the TME are predominantly Foxp3high effector Treg (eTreg) cells, the accumulation of which is linked to poor prognosis of various cancers (Wing et al., 2019). Multiple studies showed that destabilized FOXP3 in Treg cells promotes antitumor immunity and suppresses tumor development (Overacre-Delgoffe and Vignali, 2018; Cortez et al., 2020; Yang et al., 2020). Conversely, elevated Foxp3 expression in tumoral-infiltrating Treg cells suppressed the proliferation of effector T cells and promoted gastric cancer progression (Yuan et al., 2010). The molecular mechanism that maintains high Foxp3 expression in tumor-infiltrating Treg cells is still unknown.

AMP-activated kinase (AMPK) is an essential energy sensor activated by the AMP/ATP ratio (Herzig and Shaw, 2018). The activation of AMPK signaling has emerged as a controversial regulator of tumor

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development (Hardie, 2015). One study showed that AMPK could suppress lymphomagenesis by negatively regulating the Warburg effect (Faubert et al., 2013). Conversely, other studies showed that deletion of AMPK was detrimental to the growth of KrasG12Dp53f/f tumors (Eichner et al., 2019), and inhibition of AMPK activity by Compound C blocked cell cycle progression and prevented B16F1 melanoma growth (Lee et al., 2019). In addition to its effects on cancer cells, AMPK was shown to increase the antitumor activity of tumor-infiltrating CD8+ T cells by suppressing the transcription of Pdcd1 (Zhang et al., 2020). The relationship between AMPK and Foxp3 and the role of AMPK in tumor-infiltrating Treg cells remain poorly understood.

We used in vitro and in vivo experiments to analyze the role of AMPK in the tumor-promoting effects of Treg cells. Mice with Treg-specific AMPKα1 deficiency showed increased antitumor immunity and delayed tumor progression. Conditional deletion of AMPKα1 impaired the immunosuppressive capacity of Treg cells. Furthermore, AMPKα1 deficiency increased the expression of E3 ligase CHIP and promoted the ubiquitination and proteasomal degradation of FOXP3 in Treg cells. Although further studies are necessary, our results indicate that AMPK suppression in Treg cells might be an effective strategy for cancer immunotherapy.

RESULTS

AMPKα1 is upregulated in tumor-infiltrating Treg cells but is dispensable for T cell development

AMPK is a heterotrimeric complex kinase consisting of α, β, and γ subunits, the former of which is responsible for the catalytic activity of the enzyme (Steinberg and Carling, 2019). The two isoforms of AMPKα, AMPKα1, and AMPKα2, are encoded by the Prkaa1 and Prkaa2 genes, respectively (Steinberg and Carling, 2019). AMPKα1 is the predominant isoform in T cells, which also express AMPKα2 but at lower levels (Tamas et al., 2006; Mayer et al., 2008). To explore the regulation and function of AMPK signaling in tumor-infiltrating Treg cells, we monitored AMPKα1 expression in Treg cells from tumors and spleens of mice bearing B16F10 melanoma tumors. Flow cytometry revealed that the frequency of Treg cells was higher in the TME than in the spleen (Figure 1A) and that the AMPKα1 expression level was higher in Treg cells from tumors than in Treg cells from the spleen (Figure 1B).

To further examine the role of AMPKα1 in Treg cells, we generated Treg-specific AMPKα1-deficiency mice (Prkaa1fl/flFoxp3Cre/YFP, hereafter referred to as AMPKα1Treg-/-) by crossing mice bearing loxp-flanked Prkaa1 alleles with Foxp3-Cre/YFP mice (littermate controls Prkaa1+/+Foxp3Cre/YFP referred to as AMPKα1Treg+/+). We then confirmed that the AMPKα1 protein level was deleted in Foxp3-YFP+ Treg cells but not in CD4+Foxp3-YFP- Tconv cells (T conventional cells) from the AMPKα1Treg-/- mice (Figure S1A). Flow cytometry analysis showed no difference in the frequencies of Treg cells and other T cell phenotypes in the thymus between the AMPKα1Treg-/- mice and the AMPKα1Treg+/+ mice (Figure S1B–S1D), implying that AMPKα1 plays a dispensable role in the development of Treg cells.

AMPK deletion in Treg cells suppresses tumor progression

We challenged AMPKα1Treg-/- mice and AMPKα1Treg+/+ mice with B16F10 melanoma cells to explore how AMPKα1 expression in Treg cells affects tumor development. Compared with the AMPKα1Treg+/+ mice, the AMPKα1Treg-/- mice showed decreased tumor size (Figure 1C). Furthermore, the growth of the melanoma cells was largely attenuated in the AMPKα1Treg-/- mice relative to that in the AMPKα1Treg+/+ mice (Figure 1D). Moreover, AMPKα1Treg-/- mice had lower tumor weight (Figure 1E), less tumor blood vessel formation (Figure 1F), and lower frequencies of Ki-67+ proliferating cells in their tumors (Figure 1G). Similarly, AMPKα1Treg-/- mice showed decreased tumor sizes and delayed tumor progression when compared with AMPKα1Treg+/+ mice after implantation with Lewis lung carcinoma cells (LLCs; Figure S2A–S2C). These findings indicate that deficiency of AMPKα1 in Treg cells impairs tumor progression in mice.

AMPKα1 deficiency in Treg cells promotes antitumor T cell responses

Histological analysis revealed that tumor tissues from AMPKα1Treg-/- mice had more inflammatory cell infiltration than those from AMPKα1Treg+/+ mice (Figure S3A). Further analysis showed the tumors from the AMPKα1Treg-/- mice had more CD3+, CD4+, and CD8+ T cell infiltration than the tumors from the AMPKα1Treg+/+ mice (Figures 2A and 2B); however, the frequency of tumor-infiltrating B cells was
AMPKα1 is upregulated in tumor-infiltrating Tregs and promotes tumor development

(A) Representative flow cytometry images and quantification of CD4+Foxp3+ frequency in mouse spleens and tumors on day 10 after B16F10 melanoma implantation (n = 4 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(B) Representative flow cytometry images and quantification of AMPKα1 mean fluorescence intensity (MFI) in Treg cells from mouse spleens and tumors (n = 4 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(C) Representative images of tumors from AMPKα1Treg+/+ and AMPKα1Treg−/− mice on day 13 after implantation with B16F10 cells.

(D) Tumor volume in AMPKα1Treg+/+ and AMPKα1Treg−/− mice after implantation with B16F10 cells (n = 13 in AMPKα1Treg+/+ group and n = 15 in AMPKα1Treg−/− group; data are presented as mean ± SEM and analyzed by two-way ANOVA).

(E) Tumor weight in AMPKα1Treg+/+ and AMPKα1Treg−/− mice on day 13 after implantation with B16F10 cells (n = 13 in AMPKα1Treg+/+ group and n = 15 in AMPKα1Treg−/− group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(F) Representative images of immunofluorescence and quantification of CD31 intensity in tumors from AMPKα1Treg+/+ and AMPKα1Treg−/− mice on day 10 after implantation with B16F10 cells (n = 6 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test). Three or more fields per tumor were quantified. Scale bar: 100 μm.

(G) Representative images of immunofluorescence and percentage of Ki67+ cells in tumors from AMPKα1Treg+/+ and AMPKα1Treg−/− mice on day 10 after implantation with B16F10 cells (n = 5 in AMPKα1Treg+/+ group and n = 6 in AMPKα1Treg−/− group; data are presented as individual values and mean ± SD and analyzed by Student’s t test). Three or more fields per tumor were quantified. Scale bar: 50 μm.
Figure 2. Treg-specific AMPKα1 deficient mice show increased antitumor immunity

(A) Representative immunofluorescence staining images of CD3ε, CD4, and CD8α in tumor sections from AMPKα1 Treg+/+ and AMPKα1 Treg−/− mice. Scale bar: 50 μm.

(B) Cell numbers of CD3+, CD4+, and CD8+ T cells per 40× field in tumor sections from AMPKα1 Treg+/+ and AMPKα1 Treg−/− mice (n = 5 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test). Five or more fields per tumor were quantified.

(C) Numbers of CD45+ (n = 6), CD3+ (n = 6), CD4+ (n = 6), and CD8+ (n = 5) T cells per 1 × 10⁶ tumor cells from day-13 tumor tissues of AMPKα1 Treg+/+ and AMPKα1 Treg−/− mice measured by flow cytometry (data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(D) Proportions of CD45+ (n = 6), CD3+ (n = 6), CD4+ (n = 6), and CD8+ (n = 5) T cells in day-13 tumor tissues of AMPKα1 Treg+/+ and AMPKα1 Treg−/− mice analyzed by flow cytometry (data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(E) Representative FACs images of IFN-γ-producing CD8+ cells from day-13 tumor tissues of AMPKα1 Treg+/+ and AMPKα1 Treg−/− mice.

(F) Frequency and numbers of IFN-γ-producing CD8+ cells per 1 × 10⁶ tumor cells from tumor tissues of AMPKα1 Treg+/+ and AMPKα1 Treg−/− mice (n = 6 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(G) Frequency and numbers of IFN-γ-producing CD45+ cells per 1 × 10⁶ tumor cells from tumor tissues of AMPKα1 Treg+/+ and AMPKα1 Treg−/− mice (n = 6 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(H) Frequency and numbers of IFN-γ-producing CD4+ cells per 1 × 10⁶ tumor cells from tumor tissues of AMPKα1 Treg+/+ and AMPKα1 Treg−/− mice (n = 6 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).

AMPKα1-deficient Treg cells present a ‘fragile’ phenotype in TME.

Our observations of increased antitumor immunity in AMPKα1 Treg−/− mice led us to assess possible changes in the Treg populations. Intratumoral Treg cells can present a ‘fragile’ phenotype, marked by aberrant IFN-γ expression but loss of Treg signature genes, was shown to promote more efficient antitumor immunity (Lim et al., 2021; Overacre-Delgoffe et al., 2017). Next, we tested whether AMPK could influence the formation of ‘fragile’ Treg cells in TME. Several cell surface markers are related to the immunosuppressive function of Treg cells and have been exploited as cancer therapeutic targets (Ohue and Nishikawa, 2019). Analysis of these cell surface markers revealed a decrease in CD25, CTLA-4, and PD1 expression in AMPKα1-deficient Treg cells in TME compared with that in wild-type (WT) Treg cells (Figures 2A–2C). In addition, both the frequency and mean fluorescence intensities (MFIs) of IL-10, an inhibitory cytokine expressed by Treg cells, were decreased in tumor infiltrating CD8+ T cells after AMPKα1 deletion (Figures 2D and 2F). However, the frequency and numbers of tumor-infiltrating CD45+ IFNγ+ and CD4+ IFNγ+ T cells were comparable between AMPKα1 Treg+/+ mice and AMPKα1 Treg−/− mice (Figures 2G and 2H). These findings suggest that the absence of AMPKα1 in Treg cells promotes CD8+ T cells mediated antitumor immunity.

AMPKα1 maintains FOXP3 expression in Treg cells

As a key transcriptional factor, Foxp3 controls the development and functional integrity of Treg cells (Fontenot et al., 2003). Unstable FOXP3 expression promotes IFN-γ transcription in Treg cells (Yang et al., 2020). Immunofluorescence staining of FOXP3 showed similar numbers of Foxp3+ cells in tumor sections from AMPKα1 Treg+/+ and AMPKα1 Treg−/− mice (Figure 4A). Nonetheless, the FOXP3 fluorescence intensity in Foxp3+ cells from the tumors of AMPKα1 Treg+/+ mice was lower than that from the tumors of AMPKα1 Treg−/− mice (Figures 4A and 4B), indicating that AMPKα1 deficiency decreases FOXP3 protein level in the Treg cells during tumor development.
To establish the role of AMPK in Foxp3 regulation in Treg cells, we analyze the expression of yellow fluorescent protein (YFP), a reporter of transcriptional induction of Foxp3. We found that deficiency of AMPKα1 has a mild effect on the frequency of CD4+YFP+ cells and the MFI of YFP, but significantly decreased FOXP3 expression in tumor infiltrated CD4+YFP+ Treg cells (Figures 4C and 4D). These data suggest that deletion of AMPKα1 does not affect the induction of the Foxp3 gene, but it compromises the expression of the FOXP3 protein after tumor implantation.

To test whether this effect also happened in steady state, we used both GFP antibody (YFP signal can be detected by GFP antibody) and Foxp3 antibody to analyze the CD4+YFP+ and CD4+Foxp3+ Treg frequency, respectively, in the spleen of AMPKα1+/+ and AMPKα1−/− mice without tumor implantation. Both the frequency of CD4+YFP+ Treg cells and expression of YFP were comparable between both strains, whereas the frequency of CD4+Foxp3+ Treg cells and expression of Foxp3 significantly decreased in AMPKα1−/− mice (Figures 4E-4H). Therefore, both in TME and stable state, AMPKα1 is required for Foxp3 protein level, but it does not affect the survival/proportion of Treg cells.

Deficiency of AMPKα1 impairs FOXP3 protein stability

T cell receptor (TCR) signaling is required for Foxp3 expression, either through induction of Foxp3 transcription or through post-translational modification (Ono, 2020). Activation of TCR signaling by CD3/CD28 Dynabeads promoted AMPKα1 expression in AMPKα1-sufficient (WT) Treg cells but not in AMPKα1-deficient (KO) Treg cells (Figures 5A and 5B). Although FOXP3 expression was also increased in both types of Treg cells after CD3/CD28 treatment, the folds of increase in the AMPKα1-deficient (KO) Treg cells were smaller compared to the AMPKα1-sufficient (WT) Treg cells (Figures 5C and 5D). These data suggest that AMPKα1 is partially required for TCR-induced FOXP3 upregulation.

To further investigate the intracellular mechanism by how AMPKα1 regulates Foxp3 expression in Treg cells, we isolated Treg cells from AMPKα1+/+ and AMPKα1−/− mice and compared the protein and mRNA levels of Foxp3. We found that the FOXP3 protein level was much lower in the AMPKα1−/− Treg cells than those in the AMPKα1+/+ Treg cells (Figure 5E), whereas the Foxp3 mRNA level was similar (Figure 5F).

Next, we asked whether AMPK regulates FOXP3 protein stability. We transfected human embryonic kidney (HEK) 293 cells with Flag-Foxp3 plasmid together with AMPKα1 plasmid or AMPKα1 siRNA (Figures 5G and 5H). We found that AMPKα1 overexpression prevented cycloheximide (CHX)-induced reduction of FOXP3 levels (Figure 5G), whereas AMPKα1 silencing decreased the protein stability of FOXP3 (Figure 5H). These data suggest that AMPKα1 stabilizes the FOXP3 protein through post-translational modification.

Deficiency of AMPKα1 promotes FOXP3 degradation through E3 ligase CHIP

The stability of FOXP3 is tightly regulated by proteasomal degradation (Barbi et al., 2015). We therefore asked whether the reduced protein level of FOXP3 in AMPKα1-deficient Treg cells could be recovered by blocking proteasomal degradation. As shown in Figure 6A, treatment with the proteasome inhibitor MG132 normalized Foxp3 levels in Treg cells from AMPKα1−/− mice. Because post-translational ubiquitination of FOXP3 affects FOXP3 stability and Treg function (Barbi et al., 2015), we next determined whether AMPKα1 regulates FOXP3 ubiquitination. To this end, Flag-Foxp3 plasmids were transfected into HEK
293T cells together with either control siRNA or AMPKα1 siRNA. We then treated the cells with MG132 and performed Flag-Foxp3 pull-down and immunoblot to assess the ubiquitination of Foxp3. We found that ubiquitination of FOXP3 was increased when AMPKα1 was knocked down (Figure 6B). These results imply that deficiency of AMPKα1 promotes Foxp3 degradation through polyubiquitination-mediated proteasomal degradation, which results in a decrease of Foxp3 levels in Treg cells.

Finally, we investigated how AMPK regulates FOXP3 ubiquitination and degradation. The polyubiquitination and degradation of FOXP3 are controlled by E3 ligase CHIP (STUB1) and deubiquitinase USP7 (Chen et al., 2013; van Loosdregt et al., 2013). We found that the expression of deubiquitinase USP7 was comparable between AMPKα1-sufficient and AMPKα1-deficient Treg cells (Figure 6C); however, both the protein and mRNA level of CHIP were higher in AMPKα1-deficient Treg cells than those in AMPKα1-sufficient Treg cells (Figures 6D and 6E). We observed a similar effect in tumor infiltrating Treg cells, which showed increased CHIP expression after AMPKα1 deletion (Figure S4). Furthermore, CHIP knockdown by shCHIP lentivirus partially recovered the reduced Foxp3 expression in AMPKα1-deficient Treg cells (Figures 6F and 6G). These results indicated that CHIP is partially responsible for the modulation of Foxp3 protein stability by AMPKα1.

DISCUSSION

Accumulating evidence suggest that cell-intrinsic molecules that potentiate Foxp3 expression in tumor-infiltrating Treg cells can provide new targets for cancer therapy (Xiong et al., 2020; Hatzioannou et al., 2020; Cortez et al., 2020). In this study, we found that AMPKα1 is highly expressed in tumor-infiltrating Treg cells and is indispensable in these cells for FOXP3 expression and functional integrity of Treg cells in TME. Tumor-exposed Treg-specific AMPKα1 deficiency mice exhibited increased numbers of tumor-infiltrating T cells and more efficient antitumor immunity. AMPKα1 maintained the protein stability of FOXP3 while not affecting the transcription of Foxp3 in Treg cells. Furthermore, increased E3 ligase CHIP (STUB1) expression in AMPKα1-deficient Treg cells promotes the ubiquitination and proteasomal degradation of FOXP3, while decreasing FOXP3 levels in Treg cells.

AMPK signaling activates both antitumor and protumor immune responses in the TME. Activation of AMPK by its agonist, metformin, promotes the phosphorylation and degradation of PD-L1, which promotes antitumor immunity (Cha et al., 2018). Conversely, inhibition of AMPK activity in tumor-bearing mice, as well as conditional deletion of AMPKα1 in myeloid cells, improves protective T cell immunity by suppressing the immunoregulatory function of myeloid-derived suppressor cells (Trillo-Tinoco et al., 2019). In addition, AMPK inhibition reduces immunosuppressive phenotypes in tumor-associated macrophages and unleashes an antitumor effector T cell response (Xu et al., 2018; Wang et al., 2019). Thus, the modulation of AMPK has combined effects in different cell populations within the TME. The nutrient sparse, hypoxic, and acidic environment of tumor tissues negatively affect T effector cell function while supporting Treg cell function (Watson et al., 2021; Zappasodi et al., 2021). AMPK is upregulated and activated in the hypoxic and nutrient-limited TME (Gutierrez-Salmeron et al., 2020; Hao et al., 2015); however, the regulation and role of
Figure 5. AMPKα1 maintains the protein stability of FOXP3

(A) Isolated primary Treg cells (CD4+YFP+) from AMPKα1Treg+/+ (WT) and AMPKα1Treg−/− (KO) mice were treated with Dynabeads CD3/CD28 beads at 1:1 ratio for 24 h. The expression of AMPKα1 and FOXP3 were detected by Western blot.

(B) Quantification of relative AMPKα1 expression in Treg cells from AMPKα1Treg+/+ (WT) and AMPKα1Treg−/− (KO) mice stimulated with or without Dynabeads CD3/CD28 (n = 3 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(C) Quantification of relative FOXP3 expression in Treg cells from AMPKα1Treg+/+ (WT) and AMPKα1Treg−/− (KO) mice stimulated with or without Dynabeads CD3/CD28 (n = 3 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(D) Quantification of the increased FOXP3 level (ΔFoxp3) after CD3/CD28 treatment between Treg cells from AMPKα1Treg+/+ (WT) and AMPKα1Treg−/− (KO) mice (n = 3 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(E) Representative Western blot and quantification of FOXP3 protein levels in AMPKα1+/+ and AMPKα1−/− Tregs (n = 5 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(F) Quantification of relative Foxp3 mRNA levels in in AMPKα1+/+ and AMPKα1−/− Tregs (n = 6 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).
AMPK in tumor-infiltrating Treg cells are largely unknown. Our results show that AMPK upregulation in tumor-infiltrating Treg cells prevents antitumor T cell response and thus promotes tumor progression, suggesting that specific inhibition of AMPK signaling in Treg cells might have therapeutic value for cancer treatment.

Our results support AMPKα1 is required for FOXP3 protein stability and functional integrity of Treg cells in TME. Consistently with our observations, Michalek et al. reported showed that p-AMPK expression was higher in Treg cells than in conventional T cells, and that AMPK activation promoted the generation of Treg cells in vivo by regulating fatty acid oxidation (FAO) (Michalek et al., 2011). Consistent with these findings, pioglitazone and metformin were shown to enhance Treg cell expansion and inhibit the progression of plaque instability and autoimmune encephalomyelitis through activation of AMPK signaling (Sun et al., 2016; Tian et al., 2017). By contrast, other studies showed that absence of AMPKα1 and AMPKα2 had only a mild impact on immune homeostasis and the survival of Treg cells (Yang et al., 2017; Timilshina et al., 2019). The discrepancy between our results and the previous results might be because of different animal models, as the previous studies were mainly focused on the autoimmune disease caused by LKB1 and used very young mice in steady state. In addition, recent studies showed that SREBP1 or CoREST (REST corepressor 1) deletion in Treg cells could promote antitumor effects while having limited impacts on immune homeostasis in steady state (Lim et al., 2021; Xiong et al., 2020), which matches the heterogeneous results regarding AMPK deletion. The role of AMPK in Treg cells warrants further investigation.

Stabilization of Foxp3 maintains Treg lineage plasticity and hampers the antitumor immune response (Martin et al., 2010). Uncovering the fundamental regulators that control Foxp3 expression is therefore essential for understanding and exploiting efficient Treg therapies for cancer treatment (Cortez et al., 2020). Loss of Foxp3 expression transforms Treg cells into so-called ex-Treg cells, which have no immunosuppressive ability but acquire effector Th cell-like phenotypes (Hori, 2014). This has potential clinical relevance, because the accumulation of ex-Treg cells in the TME potentiates immunotherapy (Hatzioannou et al., 2020; Li et al., 2020). The decreased FOXP3 protein stability and normal Foxp3 gene induction observed in AMPKα1-deficient Treg cells in our study is not fully in accordance with the phenotype of ex-Treg cells; however, impaired FOXP3 protein stability without impairment of Foxp3 gene expression was also shown to impair Treg lineage stability and promote ex-Treg transformation in one recent study (Liu et al., 2019). It would therefore be interesting to investigate the effect of AMPKα1 deficiency on ex-Treg transformation by crossing Prkaa1Treg-/- mice with Treg lineage-tracking Foxp3GFP-REES-YFP-Cre-Rosa26-loxap-IRES-lacZ mice (Gaddis et al., 2018). Fragility of Treg cells in TME was shown to promote antitumor immunity which potentiates the therapeutic effects of immune checkpoint therapy (Hatzioannou et al., 2020; Lim et al., 2021). However, the intracellular mechanism through which intratumoral Treg cells prevent fragile IFN-γ-expressing Treg cells in TME remains largely unknown. Our findings demonstrate an essential role of AMPK in the prevention of induction of fragile Treg cells during tumor development.

FOXP3 ubiquitination and Treg function are tightly regulated by the E3 ubiquitin ligase CHIP (STUB1) (Chen et al., 2013). We found that AMPKα1 deficiency upregulated CHIP expression in Treg cells in vivo and in vitro; however, the increased CHIP expression in tumors of AMPKα1Treg−/− mice was not confined to Foxp3 Treg cells but also appeared in non-Treg cells. A previous study showed that CHIP transcription and expression were tightly correlated with inflammatory cytokines (Chen et al., 2013). Therefore, we hypothesize that the increased CHIP expression in non-Treg cells in our experiments was because of the strong inflammatory conditions in the tumors of the AMPKα1Treg−/− mice. In addition to inflammatory cytokines, CHIP transcription is linked to different stresses such as heat shock and oxidative damage (Chen et al., 2013; Paul and Ghosh, 2014; Stankowski et al., 2011). Because of the crucial role of AMPK in oxidative stress regulation, the upregulation of CHIP in AMPKα1-deficient Treg cells might be because of increased oxidative stress (Ren et al., 2020). Some post-translational modifications were also reported to regulate the expression or activity of CHIP (Paul and Ghosh, 2014; Zemanovic et al., 2018). Furthermore, one previous study showed a direct association between CHIP and AMPK levels in cardiomyocytes (Schisler et al., 2013), suggesting that another possible mechanism of CHIP regulation is direct phosphorylation of CHIP.
Figure 6. Deficiency of AMPKα1 promotes FOXP3 degradation through E3 ligase CHIP

(A) Western blot and quantitative analysis of FOXP3 in AMPKα1-sufficient (AMPKα1+/+) and AMPKα1-deficient (AMPKα1Treg-/-) Treg cells (CD4+YFP+) in the presence or absence of proteasome inhibitor MG132 (n = 3 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(B) Effect of AMPKα1 knockdown on FOXP3 ubiquitination. Flag-Foxp3 together with siControl or siAMPKα1 was transfected into HEK293T cells. The cells were then treated with 5 μM MG132 for 4 h before harvest and lysis. Ubiquitination of FOXP3 proteins was detected by Western blot.

(C) Western blot analysis of AMPKα1, USP7, CHIP, and FOXP3 protein levels in sorted AMPKα1-sufficient (AMPKα1Treg+/+) and AMPKα1-deficient (AMPKα1Treg-/-) Treg cells (CD4+YFP+).

(D) Quantification of relative CHIP expression in sorted AMPKα1-sufficient (AMPKα1Treg+/+) and AMPKα1-deficient (AMPKα1Treg-/-) Treg cells (CD4+YFP+) (n = 3 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(E) Quantification of relative CHIP mRNA levels in sorted AMPKα1-sufficient (AMPKα1Treg+/+) and AMPKα1-deficient (AMPKα1Treg-/-) Treg cells (CD4+YFP+) (n = 6 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).

(F) Western blot analysis of FOXP3 protein levels in AMPKα1-sufficient (AMPKα1Treg+/+) and AMPKα1-deficient (AMPKα1Treg-/-) Treg cells (CD4+YFP+) in the presence of shControl lentivirus or shCHIP lentivirus.

(G) Quantification of relative FOXP3 expression in AMPKα1-sufficient (AMPKα1Treg+/+) and AMPKα1-deficient (AMPKα1Treg-/-) Treg cells (CD4+YFP+) in the presence of shControl or shCHIP lentivirus (n = 4 in each group; data are presented as individual values and mean ± SD and analyzed by Student’s t test).
by AMPK. Although the E3 ligase function of CHIP is well known, the mechanism by which CHIP itself is regulated is much less clear. Further experiments are needed to determine how AMPK regulates CHIP in Treg cells.

In conclusion, AMPKα1 is a critical mediator controlling FOXP3 stability and the functional integrity of Treg cells in TME. Further studies are warranted to investigate whether therapeutic inhibition of AMPK activity in Treg cells bolsters antitumor immunity.

Limitation of the study
In this study, we showed that AMPKα1 promotes tumor development through elevation of the protein stability of Foxp3 in Treg cells. Mechanistically, AMPKα1 downregulates CHIP, a well-known E3 ligase of Foxp3 which prevents Foxp3 ubiquitination and degradation. However, this specific mechanism on how AMPK regulates CHIP expression in Treg cells still lacks exploitation. It is meaningful to further investigate the detailed mechanism on how AMPK regulates CHIP expression in Treg cells. In addition, the exploitation of AMPK’s role in Treg cells in clinical value needs to be further studied.

STAR METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103570.

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AUTHOR CONTRIBUTIONS
J.A., P.S., and M.-H.Z. designed the experiments. J.A., C.Y., J.L., and S.Y. carried out all the experiments. J.A. and P.S. wrote the manuscript and prepared figures and tables. Z.L. constructed the pCHD1-Flag-SBP-Foxp3 plasmid. J.A., Y.D., Z.L., and S.Y., analyzed the data. M.-H.Z. conceived the project and revised the manuscript. All authors had final approval of the submitted and published version.

DECLARATION OF INTERESTS
The authors declare no conflicts of interest.
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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**       |        |            |
| Flow cytometry: PE anti-mouse CD45 antibody (30-F11)  | BioLegend | 103106, RRID: AB_312971 |
| Flow cytometry: Pacific blue anti-mouse CD45 antibody (30-F11) | BioLegend | 103126, RRID: AB_493535 |
| Flow cytometry: Alexa Fluor 700 anti-mouse CD45 antibody (30-F11) | BioLegend | 103128, RRID: AB_493715 |
| Flow cytometry: Brilliant Violet 605™ anti-mouse CD3e antibody (17A2) | BioLegend | 100237, RRID: AB_2562039 |
| Flow cytometry: Pacific blue anti-mouse CD4 antibody (RM4-5) | BioLegend | 100531, RRID: AB_493374 |
| Flow cytometry: PE/Cyanine 7 anti-mouse CD4 antibody (GK1.5) | BioLegend | 100422, RRID: AB_312707 |
| Flow cytometry: Alexa Fluor 488 anti-mouse CD8a (S3-6.7) | BioLegend | 100723, RRID: AB_389304 |
| Flow cytometry: Alexa Fluor 488 anti-mouse/ rat/human Foxp3 antibody (150D) | BioLegend | 320012, RRID: AB_439748 |
| Flow cytometry: PE anti-mouse Foxp3 antibody (MF-14) | BioLegend | 126404, RRID: AB_1089117 |
| Flow cytometry: Alexa Fluor 488 anti-mouse Foxp3 antibody (MF-14) | BioLegend | 126406, RRID: AB_1089114 |
| Flow cytometry: Alexa Fluor 488 anti-GFP (FM264G) | BioLegend | 338008, RRID: AB_2563288 |
| Flow cytometry: PE anti-GFP(FM264G) | BioLegend | 338004, RRID: AB_2650615 |
| Flow cytometry: PE Rat anti-mouse CD25(3C7) | BioLegend | 101904, RRID: AB_312847 |
| Flow cytometry: PE CD278(COS) (15F9) | BioLegend | 107705, RRID: AB_313334 |
| Flow cytometry: PE anti-mouse IFN-γ(XMG1.2) | BioLegend | 505808, RRID: AB_315402 |
| Flow cytometry: PE-Cy7 anti-mouse CD357 (GITR) (YGITR 765) | BioLegend | 120222, RRID: AB_528907 |
| Flow cytometry: PE anti-mouse CD152 (CTLA-4) (UC10-4B9) | BioLegend | 106305, RRID: AB_313254 |
| Flow cytometry: PE anti-mouse CD279 (PD1) (RMP1-30) | BioLegend | 109103, RRID: AB_313420 |
| Flow cytometry: PE anti-mouse IL10 (JESS-16E3) | BioLegend | 505008, RRID: AB_315362 |
| Flow cytometry: PE anti-mouse CD304 (Nrp1) (3E12) | BioLegend | 145204, RRID: AB_2561928 |
| Western blot: Rabbit anti-FOXP3 antibody | abcam | Ab75763, RRID: AB_1310238 |
| Western blot: FOXP3 Monoclonal Antibody (150D/E4) | eBioscience | 14-4774-82, RRID: AB_467552 |
| Immunofluorescence: FOXP3 (D608R) rabbit | Cell Signaling | 126535, RRID: AB_2797979 |
| Flow cytometry/Western blot/ Immunohistochemistry: Recombinant anti-AMPK alpha 1 antibody | Abcam | ab32047, RRID: AB_722764 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Immunohistochemistry:** Recombinant anti-AMPK alpha 1 antibody | Abcam | Ab110036; RRID:AB_10862578 |
| **Western blot:** Goat AMPKa1(C-20) | Santa Cruz | Sc-19128; RRID:AB_2268724 |
| **Immunofluorescence:** CD3e Monoclonal antibody (SP7) | Thermo Fisher | MAS-14524; RRID:AB_10982026 |
| **Immunofluorescence:** Recombinant anti-CD8 alpha antibody | Abcam | Ab217344; RRID:AB_2890649 |
| **Immunofluorescence:** Recombinant anti-CD4 antibody | Abcam | Ab183685; RRID:AB_2686917 |
| **Immunofluorescence:** CD31(PECAM-1) (D8V9E) Rabbit mAb | Cell signaling | 77699; RRID:AB_2722705 |
| **Immunofluorescence:** Recombinant anti-Ki-67 antibody (SP6) | Abcam | Ab16667; RRID:AB_302459 |
| **A Western blot:** Anti β-actin antibody(C4) | Santa Cruz | Sc-47778; RRID:AB_2714189 |
| **Western blot/Immunofluorescence:** Anti-ChIP(Stub1) antibody | Santa Cruz | Sc-133066; RRID:AB_2286870 |
| **Western blot:** Anti-HAUSP(USP7) antibody (H-12) | Santa Cruz | Sc-137008; RRID:AB_2214163 |
| **Western blot:** Ubiquitin antibody | Cell signaling | 3933; RRID:AB_2180538 |
| **Immunofluorescence:**CD45R(B220) Mouse Monoclonal Antibody (RA3-6B2) | Thermo Fisher | 14-0452-85; RRID:AB_467255 |
| **Flow cytometry/Immunofluorescence:** Goat anti-Rabbit IgG H&L (Alexa Fluor 488) | abcam | Ab150077; RRID:AB_2630356 |

**Chamicals, peptides, and recombinant proteins**

| Chemical | Source | Identifier |
|----------|--------|------------|
| Anti-CD3/CD28 Dynabeads | ThermoFisher | 11452D |
| IL2 Recombinant Mouse Protein | ThermoFisher | PMC0025 |
| Fixable Viability Dye eFluor 660 | ThermoFisher | 65-0864-18 |
| Fixable Viability Dye eFluor 780 | ThermoFisher | 65-0865-14 |
| Cycloheximide | SIGMA | 01810 |
| MG132 | SIGMA | 133407-82-6 |
| DNase | SIGMA | 10104159001 |
| Collagenase D | SIGMA | 11088866001 |
| iScript cDNA Synthesis Kit | BioRad | 170-8891 |
| Lipofectamine 2000 Transfection Reagent | ThermoFisher | 11668019 |
| Lipofectamine RNAiMAX Transfection Reagent | ThermoFisher | 13778075 |
| Albumin, Bovine Fraction V (BSA) | RPI | A30075-100;CAS.9048-46-8 |
| Fetal bovine serum (FBS) | Sigma-Aldrich | 12303C |
| Penicillin-Streptomycin | ThermoFisher | 15140122 |
| RPMI 1640 | CORNING | 10-040-CV |
| DMEM | CORNING | 10-013-CV |

**Critical commercial assays**

| Assay | Source | Identifier |
|-------|--------|------------|
| Dynabeads Mouse CD4⁺ CD25⁺ Treg isolation Kit | ThermoFisher | 11463D |
| eBioscience Foxp3/Transcription Factor Staining Buffer Set | ThermoFisher | 00-5523-00 |
| eBioscience Protein Transport Inhibitor Cocktail (500x) | ThermoFisher | 00-4980-03 |
**RESOURCE AVAILABILITY**

**Lead contact**
Requests for further information and reagents may be directed to the lead contact, Ming-Hui Zou (mzou@gsu.edu).

**Materials availability**
Unique reagents generated in this study are available from the lead contact with a completed Material Transfer Agreement.

**Data and code availability**
All data produced in this study are available from the lead Contact upon request.

This paper has no original code.

Any additional information required to reanalyze the data showed in this paper is available from the lead Contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Cell lines**
HEK 293T cells, B16-F10-luc2 cells, and LLC cells were cultured in Dulbecco’s Modification of Eagle’s Medium (CORNING) containing 4.5 g/L glucose, L-glutamine, and sodium pyruvate, supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 37°C incubator with humidity and 5% CO2. Moreover, primary mouse cells were isolated from 6–8 mice/group on the same day.
Mouse strains
Prkaa1fl/fl, Foxp3Cre/YFP, and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). All genetic models had the C57BL/6 background. Prkaa1fl/flFoxp3Cre/YFP mice were used at 10-16 weeks of age unless otherwise noted. Age-matched and sex-matched Prkaa1+/+Foxp3Cre/YFP mice were used as controls.

All mice were housed in specific pathogen-free conditions in the animal facilities at Georgia State University. The animal protocol was reviewed and approved by the Georgia State University Institutional Animal Care and Use Committee.

Tumor models
B16-F10 murine melanoma cells and LLC cells were purchased from the ATCC. First, we confirmed that the cell lines were negative for Mycoplasma spp. We subcutaneously implanted 5x10^5 B16-F10 melanoma cells or 5x10^5 LLC cells on the backs of 10–12-week-old male AMPKα1Treg+/+ and AMPKα1Treg−/− mice. The mice were monitored every day, and tumor growth and tumor size were determined based on tumor volume (0.5 x width^2 x length). Tumors were collected 10-13 days after inoculation unless otherwise noted.

METHOD DETAILS
Cell isolation from tumors and lymphoid organs
Tumor-infiltrating lymphocytes were isolated by incubating tumor tissues in collagenase D (1 mg/ml, Roche) and DNase I (0.25 mg/ml, Sigma) for one hour. Single-cell suspensions from tumors were generated by passing the digested tumor tissues through a 40-μm cell strainer. Single-cell suspensions from spleen were obtained directly by passing the tissues through a 40-μm cell strainer.

Flow cytometry
Each reaction was performed with more than 1 x 10^6 cells, and a minimum of 1 x 10^5 events were recorded. Fluorescence-positive cells were analyzed with a FACScalibur or LSRFortessa device (Becton Dickinson, CA). Cells were processed for viability staining using eBioscience LIVE/DEAD fixable Dye eFluor 660 or eFluor 780 (Thermofisher). For analysis of cell surface markers, cells were stained in stain buffer (BD biosciences) with the appropriate antibodies. Information on antibodies, clones, and fluorophores is provided in the Key resources table. A Foxp3/transcription factor staining buffer set from eBioscience was used for intracellular staining. For staining intracellular cytokines, cells were stimulated for 6-18 h with a cell stimulation cocktail (Thermofisher) conjunction with protein transport inhibitor cocktail (Thermofisher) before being stained. After stimulation, the cells were stained with appropriate antibodies following the manufacturer’s instructions. For YFP staining, Alexa Fluor 488 anti-GFP or PE anti-GFP antibody from BioLegend was used.

Histopathology and immunofluorescent staining
Organs were fixed with 10% (v/v) neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For immunofluorescent staining, 4-μm sectioned paraffin slides were subjected to xylene and alcohol for dehydration. Then, the slides were subjected to antigen retrieval, permeabilized with 0.2% Triton X-100, and blocked with protein block goat serum (BioGenex, Fremont, CA). Then, the slides were incubated with CD31, anti-Ki67, Foxp3, CD3e, B220, CD4, CD8, or CHIP antibodies at 4°C overnight. Alexa Fluor 555 and Alexa Fluor 488 goat anti-rabbit, anti-rat, or anti-mouse were used as secondary antibodies, incubated at room temperature for one h. Cell nuclei were stained with DAPI and mounted with VectaMountTMAQ (#5501, Vector Laboratories) for fluorescence microscopy. Quantification was performed using Image J software.

Cell purification and culture
Lymphocytes were isolated from spleens, CD4+ T cells were isolated using a Dynabeads CD4+ T cell Isolation Kit (ThermoFisher). Then, CD4+YFP+ Treg cells were purified by YFP (GFP staining) using FACSaria II (BD Bioscience). The purified cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and β-mercaptoethanol.
**RNA extraction and qRT-PCR**

According to the manufacturer’s instructions, RNA was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA was synthesized using the iScript cDNA Synthesis Kit. The expression of Foxp3 and CHIP mRNAs was determined by quantitative real-time PCR (qRT-PCR). Each cDNA sample was amplified using SYBR Green (Bio-rad, Hercules, CA) on a Bio-rad CFX96 Touch Real-time PCR detection system.

**Western blot analysis**

Cells were lysed with NP-40 lysis buffer (1% NP-40, 20 mM Tris-Cl pH 7.5, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 2 mM Na$_3$VO$_4$, and 10 μg/ml each of aprotinin and leupeptin) or with 1× SDS sample buffer (50 mM Tris-Cl pH 6.8, 100 mM DTT, 2% SDS, and 10% glycerol). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Billerica, MA). Membranes were visualized with an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Waltham, MA). When necessary, the membranes were stripped by incubation in stripping buffer (Thermo Fisher Scientific, Waltham, MA) for 15 - 30 min with constant agitation, washed, and then re-probed with various other antibodies.

**Immunoprecipitation**

For immunoprecipitation, HEK293T cells were transfected with lipo2000 or RNA iMAX. Forty-eight hours after transfection, the cells were treated with MG132 for six h and then lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 0.5% Nonidet p40 (US Biological), 1 mM EDTA and 40 mM NaCl with 1% protease inhibitor cocktail (ThermoFisher)). Anti-FLAG M2 affinity gel was used for immunoprecipitation. The precipitated samples were then boiled and analyzed by western blot.

**Lentiviral shRNA transduction**

CHIP (Stub1) shRNA(m) lentiviral particles were obtained from Santa Cruz. Purified CD4$^+$YFP$^+$ Treg cells were activated with Dynabeads CD3/CD28 plus IL-2 (200 U/ml) for 48 h and then transduced with lentivirus in the presence of 10 μg/ml polybrene by centrifugation at 900 g for 3 h. The transduced cells were then cultured in RPMI 1640 medium for 48 h and subsequently lysed for western blot analysis.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, CA). Student’s t test was used for analysis comparing two samples. One-way ANOVA tested differences among multiple samples. Two-way ANOVA was applied to study the effect of two parameters (i.e., time and treatment) and their interaction. P < 0.05 was considered statistically significant.