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FADD is essential for glucose uptake and survival of thymocytes

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ABSTRACT

Fas-associated protein with death domain (FADD) has been implicated in T lymphocytes, but the nature of FADD-dependent mechanism in early T cell development has not been completely elucidated. In this study, using T-cell specific deletion mice, we observed that FADD deficiency in thymocytes led to increased apoptosis and reduced cell numbers, which may be attributed to the reduction of Glut1 expression and correspondingly decreased glucose uptake. Furthermore, an abnormal transduction of Akt signaling was discovered in FADD$^{-/-}$ thymocytes, which may be responsible for the declined Glut1 expression. Collectively, our results demonstrate the new function of FADD in glucose metabolism and survival of early T cells.

1. Introduction

T cells develop in thymus through a series of tightly regulated steps. The thymic immature CD4$^-$CD8$^-$ double-negative (DN) T cells develop into CD4$^+$CD8$^+$ double-positive (DP) and then to CD4$^+$CD8$^-$ single-positive (CD4SP) or CD4$^-$CD8$^+$ (CD8SP) thymocytes [1]. Several checkpoints are involved in the differentiation and mature of thymocytes. At the DN stage, a properly rearranged TCRβ chain pairs with pre-TCRα (pTα) to form the pre-TCR, which signals in a ligand-independent manner to drive proliferation and differentiation to the DP stage [2]. Then a mature αβTCR on the cell surface is needed for the DP cells to pass through positive selection and negative selection [3]. Most thymocytes that fail to signal through the pre-TCR or TCR will die by apoptosis.

Glucose metabolism is found to be crucial to the survival, differentiation, and proliferation of developing T cells in the thymus [4], in which the phosphoinositol-3 kinase (PI3K)-Akt pathway plays an important role in regulation. Activated Akt can protect cells against various death stimuli by promoting glucose uptake and metabolism [5–7]. Glut1, the major glucose transporter in peripheral T lymphocytes, appears to be the main functional glucose-transporter isoform in T lymphocytes [8]. In thymus, Glut1 expression varies among subsets, showing different metabolic properties of thymocytes in corresponding development stages [9,10]. Akt is suggested to regulate glucose metabolism through the combined increases in Glut1 surface expression and activity of the glucose transporter [11]. Ablation of Akt isoforms in thymocytes leads to decreased glucose uptake and impaired cell survival or differentiation [12–14].

Fas-associated protein with death domain (FADD) is considered as an important molecule regulating death and living pathways during different kinds of biological events, such as apoptosis, cell cycle progression, inflammation, and innate immunity [15–17]. Recent work also found that FADD phosphorylation regulated glucose homeostasis in liver tissue [18]. The function of FADD in early T cell development has been investigated for a long time. However, the detailed mechanism of how FADD regulates T cell development still remains unraveled. In this article, we generated T cell-specific FADD knockout mice and demonstrated that FADD deficiency led to an obvious loss of cell numbers. Meanwhile, a reduction of glucose uptake and decreased Glut1 expression was observed, which may account for the increased apoptosis in FADD$^{-/-}$ thymocytes. In addition, an abnormal transduction of Akt signaling was found in FADD$^{-/-}$ thymocytes, suggesting that the expression of Glut1 and regulation of glucose uptake may be controlled by FADD through Akt-dependent pathways. Taken together, these findings demonstrated that FADD may participate in glucose metabolic regulation in thymocytes and promote cellular survival in early T cell development.

Abbreviations: FADD, Fas-associated protein with death domain; DN, double negative; DP, double positive; SP, single positive; TCR, T cell receptor; MEFs, mouse embryo fibroblasts; 2-NBDG, 2-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose.

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2. Materials and methods

2.1. Mice

The generation and genotypes of FADD$^{lox/lox}$ Lck-Cre Mice were assessed as previously described [19]. The mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care Jiangsu Province credited SPF animal facility in Nanjing Drum Tower Hospital and all animal protocols are approved by the Animal Care and Use Committee of the School of Life Sciences of Nanjing University.

2.2. Cell culture and transfection

FADD$^{+/-}$ or FADD$^{-/-}$ MEFs were cultured in Dulbecco Modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and transfected using Amaxa Nucleofector (Lonza) according to the manufacturer's instructions.

2.3. Protein preparation and immunoblotting

Protein extracts were prepared and subjected to western blot analysis as described [4].

2.4. Immunofluorescence microscopy

Unfixed thymus was embedded in OCT compound (Sakura), cut, fixed in acetone, and stained as previously described [18]. Secondary reagent used was Alexa 594-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). Nuclear DNA was stained using 4′,6-diamidino-2-phenylindole (DAPI). Images were then visualized by microscopy (Carl Zeiss Axioplan 2).

2.5. Abs and flow cytometry analysis

All Antibodies (Abs) were from eBiosciences. For flow cytometric analysis, the following Abs were used: anti-CD4 (GK1.5), anti-CD8 (53–6.7), anti-CD25 (PC61.5), anti-CD44 (IM7). Abs were all APC, FITC, PE or PE-cy5 conjugates. For staining, cells were plated in tubes at a density of 5×10^5–1×10^6 cells per sample. Fluorochrome-conjugated Abs were added to cells and incubated on ice for 30 min. Cells were washed three times, and analyzed by flow cytometry on a FACS Calibur (BD Biosciences) flow cytometer using WinMDI software.

2.6. Quantitative RT-PCR

DNA or mRNA was isolated from Thymocytes populations. cDNA generated with SuperscriptII was amplified and used for quantitative real-time RT-PCR analyses for Bcl-xL, Bak1 and Bax, as well as β-actin as an internal control. An ABI RT-PCR System was used, and error was calculated via the 2-ΔΔCT method.

2.7. Apoptosis detection with TUNEL staining

Isolated thymocytes were stained using the APO-DIRECT™ Kit (BD Pharmingen) according to the instructions and then analyzed by flow cytometry on a FACS Calibur (BD Biosciences) flow cytometer using WinMDI software.

2.8. Quantitation of apoptosis by Annexin V staining

Thymocytes were incubated in RPMI complete medium at a concentration of 2×10^6 cells/ml in 24-well plates. Cells were analyzed either directly after isolation (time 0) or after incubation for the indicated time points as described [20].

2.9. Glucose uptake assays

2×10^6 thymocytes were incubated for 10 min in 500 μl glucose-free media. 500 μl glucose-free media containing 100 μM 2-NBDG (Invitrogen) was added and incubated for 30 min before measuring fluorescence by flow cytometry.

2.10. Electron microscopy

1×10^6 thymocytes were pelleted and fixed on ice for 30 min in 0.1 M NaCacodylate, pH 7.4, containing 2% Glutaraldehyde and 1% PFA. Randomly chosen fields were viewed with a Jeol 1200EX Biosystem TEM (JOEL, Peabody).

2.11. Plasmid and Abs

Plasmid of FADD was constructed as described [21]. The antibodies used in this study were as follows: anti-Glut1 (EPR3915, Epitomics, Burlingame, CA, USA), anti-FADD (EPR5030, Epitomics), anti-cleaved Notch1 (Val1744) (D3B8, Cell Signaling Technology), anti-Akt (C67E7, Cell Signaling Technology), anti-pAkt (Ser473) (D9E, Cell Signaling Technology), anti-ERK (137F6, Cell Signaling Technology), anti-pERK (Thr202/Tyr204) (D13.14.4E, Cell Signaling Technology), anti-mTOR (7C10, Cell Signaling Technology), anti-pmTOR (Ser2448) (D9C2, Cell Signaling Technology), anti-PKC-α (Cell Signaling Technology), anti-PKC-θ (E1I7Y, Cell Signaling Technology), and anti-α-Tubulin (EPR1333, Epitomics).

2.12. Statistical analysis

All quantitative data in this study were presented as mean ± S.D. A two-tailed Student's *t*-test was used to analyze comparisons between two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. FADD deficiency in thymocytes induces increased apoptosis

The floxed FADD mice were bred to Lck-cre transgenic mice, which initiate deletion as early as the DN2 stage of T cell development. With an efficient T cell-specific deletion of FADD (Fig. S1), Lck-FADD mice had a significant reduction in thymic cellularity (Table 1). The decreased number of thymocytes seen in the absence of FADD suggested that FADD may be required for cellular survival or expansion. TUNEL staining of thymocytes showed increased apoptosis in Lck-FADD mice (Fig. 1A). To confirm this observation, we analyzed the apoptotic death in cells cultured ex vivo for various time points and found that thymocytes from Lck-FADD mice were more prone to undergo apoptosis than WT controls in DP

Table 1
Effect of thymocytes FADD deficiency on total and subset cell numbers.

Cell numbers	Mice	
	WT	Lck-FADD
Total thymocytes ($\times 10^8$)	2.06 ± 0.78	0.21 ± 0.07***
DN thymocytes ($\times 10^7$)	1.04 ± 0.22	0.38 ± 0.09**
DP thymocytes ($\times 10^7$)	14.51 ± 2.64	0.82 ± 0.4***
CD4$^+$ SP thymocytes ($\times 10^7$)	2.77 ± 0.38	0.31 ± 0.09***
CD8$^+$ SP thymocytes ($\times 10^7$)	0.86 ± 0.18	0.13 ± 0.03***

Values are mean ± SEM ($n = 5$ per group).
** $P < 0.01$ versus WT controls.
*** $P < 0.001$ versus WT controls.

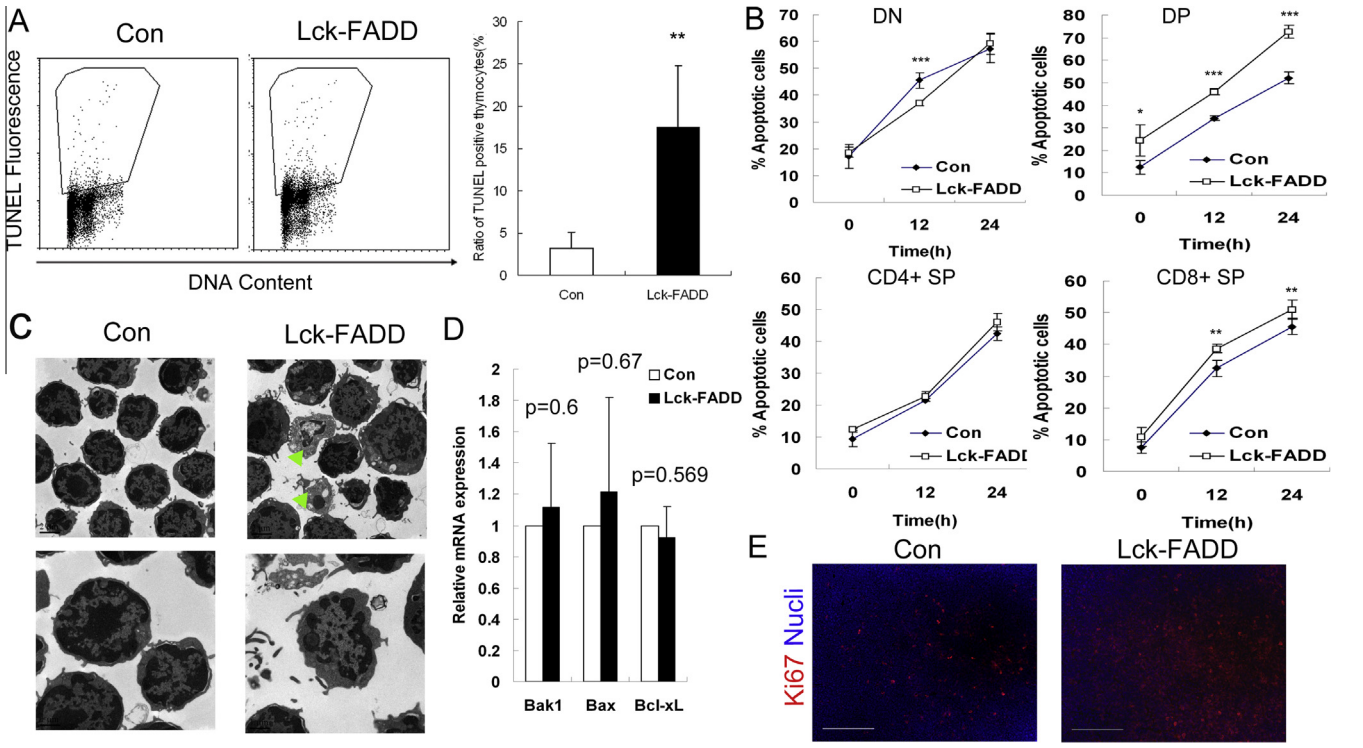

Fig. 1. FADD deficiency leads to increased apoptosis in Lck-FADD mice. (A) Thymocytes were stained with TUNEL kit and analyzed by flow cytometry to detect apoptosis in the thymus of Lck-FADD mice and littermate control. Shown is representative data from three independent experiments. Error bars reflect SEM. $^{**}P < 0.01$. (B) Thymocytes from WT or Lck-FADD mice were cultured in complete tissue culture medium for the indicated time points, after which the rate of apoptosis was assessed by flow cytometry as indicated in Section 2. Data are the mean ± the SD of values obtained from five independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (C) Electron microscopy images showing apoptosis (arrowheads) in isolated thymocytes from Lck-FADD and WT mice. Scale bars: 2 μm (up); 1 μm (below). (D) mRNA was generated and examined by quantitative PCR for Bak1, Bax and Bcl-xL mRNA expression levels, and normalized relative to the wild-type cells of each gene (=1). The data shown is from three Lck-FADD mice and littermate control. Error bars reflect SEM. (E) Cryosections of thymi were stained with antibodies and analyzed by immunofluorescence microscopy to detect the expression of Ki67 in the thymus of Lck-FADD mice and littermate control. Scale bars: 100 μm.

subset, with values being already higher at 0 h after isolation and showing an extremely significant difference after 12 h in culture (Fig. 1B). Thymocytes with the typical signatures of apoptosis were also observed by electron microscopy (Fig. 1C), while no obvious necrotic cells were detected (Fig. 1C and Fig. S2), indicating that increased apoptosis caused by FADD deficiency may be a main reason for the reduction of cell number in Lck-FADD thymus. The increased apoptosis in temporally Lck-driven thymus-specific FADD deficient T cells is contradictory to our understanding of typical function and mechanism of FADD in apoptosis. This observation forces us to check its potential involvement of FADD in the intrinsic or mitochondrial apoptosis pathway, in which Bcl-2 family is the key regulator. The 'prosurvival' Bcl-2 family members are suggested to play an important role in promoting survival of early T-cells [22]. However, as shown in Fig. 1D, there were no important differences in the transcripts of Bcl-xL gene products between thymocytes from Lck-FADD mice and WT controls. Additionally, the increased apoptosis of thymocytes was not due to the increased transcription of proapoptotic Bcl-2 family gene products, as Q-PCR analysis showed no notable differences in the expression of Bak and Bax (Fig. 1D). Meanwhile, the immunofluorescence result showed that the expression of Ki67 in the thymus of Lck-FADD mice was at least not less than that in WT controls (Fig. 1E), indicating that cell loss seen upon FADD deletion may be not due to defective proliferation in thymocytes.

3.2. Decreased expression of Glut1 and reduced glucose uptake in $FADD^{-/-}$ thymocytes

The increased apoptosis and reduced cell numbers in $FADD^{-/-}$ thymus resembled the outcome of insufficient growth factors, which influence cell survival and mitochondrial homeostasis through the maintenance of glucose metabolism [23]. To address whether cellular metabolism was affected after loss of FADD signaling, we determined the glucose metabolism in thymocytes from Lck-FADD mice and WT controls. We found that FADD deficiency led to a decrease in glucose metabolism, noted as reductions in the glucose uptake of thymocytes in vitro (Fig. 2A). When comes to subsets, the DP cells in Lck-FADD mice showed a decreased glucose uptake compared with controls (Fig. 2B), while the rates in DN, CD4SP or CD8SP cells from the two mice were similar (Fig. S2). Correspondingly, the expression of Glut1, one of the most important glucose transporters in T cells, was found declined in the thymocytes from Lck-FADD mice (Fig. 2C and D). In order to find whether FADD deficiency abrogates the expression of Glut1, we have overexpressed FADD in WT and $FADD^{-/-}$ MEFs and measure Glut1 content. As shown in Fig. 2E, Glut1 expression in $FADD^{-/-}$ MEFs was elevated and close to the level in WT cells when exogenous FADD was added, suggesting that FADD deficiency may be a reason for decreased Glut1. The data indicates that FADD also plays an important role in glucose metabolism as previously reported in liver tissue [18]. However, unlike the result of liver tissue from FADD-D mice, the phosphorylation and expression of glycogen synthase kinase (GSK) 3β, a key negative regulator in glycogen synthesis, showed no differences between Lck-FADD mice and WT controls (Fig. 2F), which exhibited the diversity of both the functions and mechanisms of FADD protein in different tissues.

3.3. FADD deletion results in abnormal activation of Akt signaling

Previous work has demonstrated that Notch1 was involved in the maintenance of cellular metabolism of thymocytes by

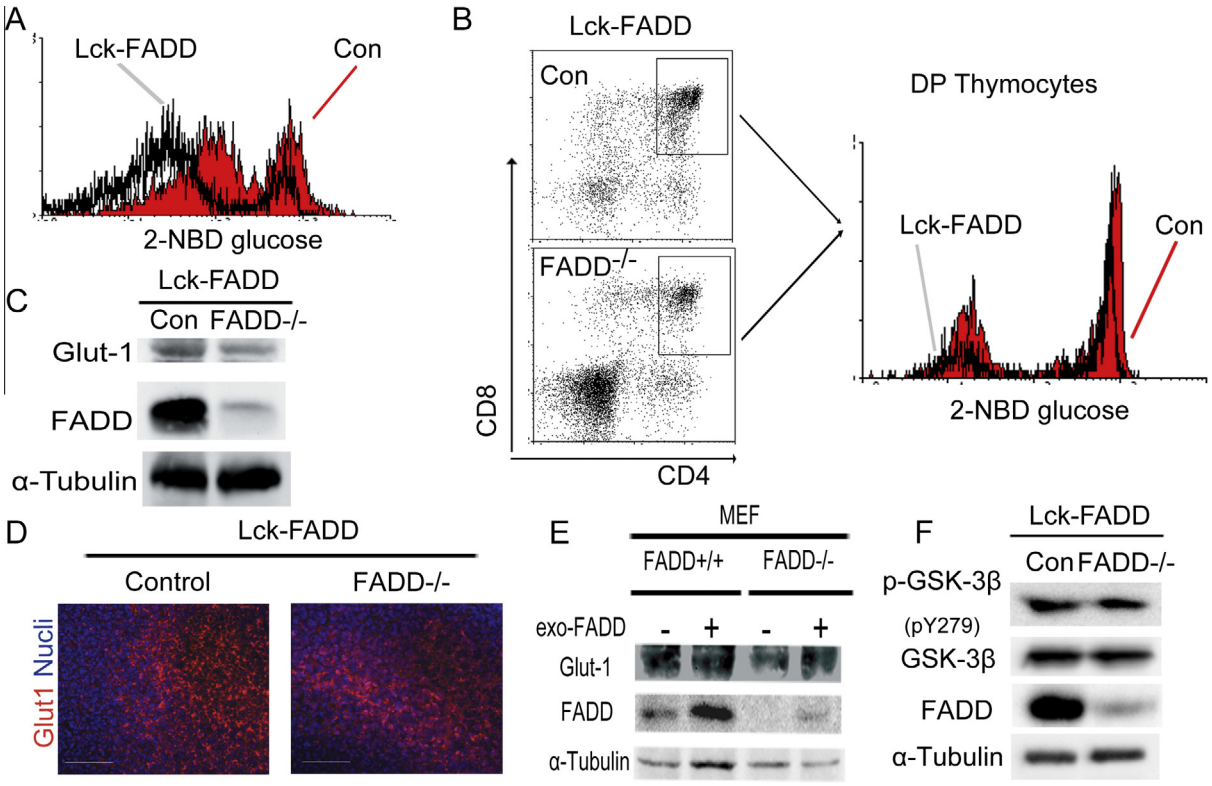

Fig. 2. Reduced glucose uptake and decreased Glut1 expression in FADD$^{-/-}$ thymocytes. (A) Representative histograms of 2-NBD glucose uptake in thymocytes cultured for 30 min. Shown is representative data from three independent experiments. (B) Flow cytometry analyses of 2-NBD glucose uptake in DP thymocytes (CD4$^+$CD8$^+$) from Lck-FADD and WT mice cultured for 30 min. (C) Western-blot analysis of Glut1 levels in thymocytes obtained from Lck-FADD mice and littermate control. The α-Tubulin expression served as a loading control. (D) Glut1 immunofluorescence analyses was performed on the thymus of Lck-FADD mice and littermate control. Scale bar: 50 μm. (E) Immunoblot analyses of Glut1 in WT and FADD$^{-/-}$ MEF cells after exogenous FADD was introduced for 24 h. (F) Western-blot analyses of the phosphorylation and expression of glycogen synthase kinase (GSK) 3β in thymocytes.

activation of the PI3K-Akt pathway [4]. The cleaved smaller cytoplasmic subunit of Notch1 was accumulated in FADD$^{-/-}$ thymocytes (Fig. 3A), suggesting that Notch1 signaling was overactivated by FADD deficiency and may lead to the activation of Akt pathway. As it was suggested that PI3K/Akt and MEK/ERK pathways are both involved in the regulation of Glut1 expression [24,25], we investigated the expression of p-Akt and p-ERK in the thymocytes from Lck-FADD mice and WT controls. Western blot result showed an obvious increase in the phosphorylation of Akt at serine 473 (Fig. 3B), while the p-ERK level was similar in the thymocytes from two mice (Fig. 3C). In order to find the effect of FADD deficiency on Akt signaling pathway, we investigated the level of mTOR phosphorylation, which was suggested as a downstream effector of PI3K-Akt signaling cascade in the regulation of Glut1 expression. However, as shown in Fig. 3D, no significant differences of mTOR phosphorylation at serine 2448 were observed between Lck-FADD mice and WT controls, suggesting that the signal transduction mediated by increased p-Akt in FADD$^{-/-}$ thymocytes was blocked, which may be responsible for the reduced expression of Glut1. Interestingly, we found that protein levels of PKC-α, which has been suggested as an inhibitor of Akt activation, was increased in FADD$^{-/-}$ thymocytes as compared with WT controls. In contrast, the expression of PKC-θ was similar in the two mice (Fig. 3E). These results suggested that PKC-α may be involved in the down-regulation of Glut1 expression in FADD$^{-/-}$ thymocytes.

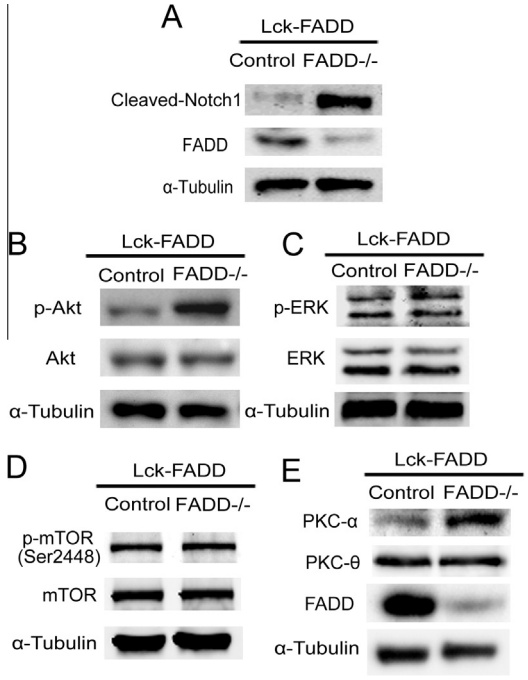

Fig. 3. Abnormal phosphorylation of Akt in FADD$^{-/-}$ thymocytes. (A) Expression of cleaved Notch1 in thymocytes was analyzed by Western blotting. (B, C) Thymocytes from Lck-FADD mice and WT littermates were lysed and immunoblotted to determine the phosphorylation levels of Akt and ERK. (D) Western blotting analyses of mTOR expression and phosphorylation in thymocytes from Lck-FADD and WT mice. (E) Immunoblot analyses of PKC-α and PKC-θ in thymocytes from Lck-FADD and WT mice.

4. Discussion

Although there are in-depth understandings about FADD-mediated apoptotic signaling pathway, relatively little is known about

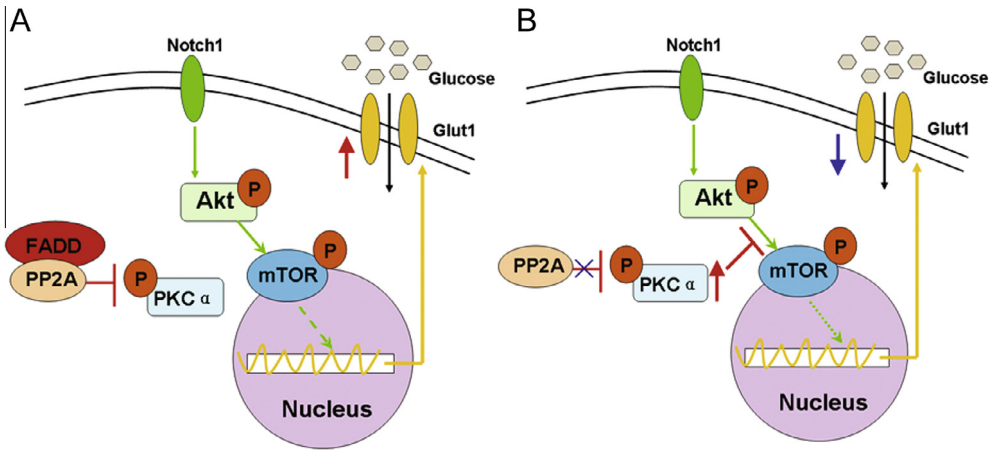

Fig. 4. Proposed mechanism of repressed glucose uptake upon inhibition of Akt signaling caused by FADD deficiency. (A) Notch signaling induces increased Glut1 transcription and promotes glucose uptake mediated by Akt phosphorylation and enhanced Glut1 expression in thymocytes. (B) Silence of FADD leads to an accumulation of PKC-α, which may inhibit signal transduction mediated by increased p-Akt and resulted in repressed Glut1 expression and glucose uptake.

the nature of FADD-dependent atypically apoptotic function. Regulating glucose homeostasis is a novel function of FADD found by proteomic discovery and physiological validation. Using MEFs and transgenic mice bearing phosphorylation-mimicking mutation form of FADD (FADD-D), glycogen synthesis, glycolysis, and gluconeogenesis were found dysregulated because of FADD phosphorylation [18]. However, whether FADD is involved in glucose metabolism during the early T cell development has not been studied. In this study, we generated T cell-specific FADD knockout mice in which FADD gene was temporally and tissue-specifically silenced from the DN2 stage of thymocytes and found all subsets in Lck-FADD mice showed reduced cell numbers, which was similar with previous discoveries [19]. An increased apoptosis was observed, especially in DP subset, indicating that FADD deficiency may cause a severe arrest for thymocytes to survival. However, the similar expression of Bcl-2 family members in $FADD^{-/-}$ and WT thymocytes suggested that the increased apoptosis may be due to neither downregulation of prosurvival genes nor upregulation of proapoptotic genes. In our previous research, we found that FADD deficiency led to increased apoptosis in DN4 cells and may result in development arrest between DN and DP stages, while normal proliferation was observed in DP cells [26]. However, whether the silence of FADD affects the metabolism and survival of DP cells remains unclear. In this article, we detected increased apoptosis correlated with decreased glucose uptake in DP cells. Therefore, our findings demonstrate that, besides a defect in the transition of DN thymocytes to the DP stage, an increase in the rate of apoptosis induced by down-regulation of Glut1 expression and glucose uptake may also account for the loss of DP cells in Lck-FADD mice, which means FADD plays an important role in regulating glucose metabolism of thymocytes.

Signals transmitted through either TCR or cytokine receptors promote cellular survival by increasing nutrient uptake and utilization correlated with increased surface expression of Glut1 [27,28]. Although some studies suggest that Akt only regulates Glut1 cell surface trafficking in response to TCR or cytokine signaling, Akt phosphorylation and activation is found consistent with the increased Glut1 expression in thymocytes [4]. The Akt/TSC/mTOR/S6K signaling pathway is suggested to be one of the mechanisms underlying the up-regulation of Glut1 expression in vascular smooth muscle cell [25]. The same mechanism may also play a role in the regulation of Glut1 expression in thymocytes. Our findings demonstrate that FADD deficiency leads to an up-regulation of Akt phosphorylation in thymocytes. It has been stated that Akt phosphorylation cannot be observed in DP thymocytes in response to TCR activation [14]. Thus, we speculate that high content of p-Akt in $FADD^{-/-}$ thymocytes may result from activated Notch1 signaling pathway, which was considered to indirectly regulate Akt via Hes1 suppression of PTEN [29]. However, unlike that stated in the previous work [29,30], no significant differences of mTOR phosphorylation were observed between Lck-FADD mice and WT controls, suggesting that the Notch1 signal in $FADD^{-/-}$ thymocytes was blocked. Therefore, there may exist other factors interfering in the signal passage from Akt to downstream effectors.

Recently, our laboratory has found that FADD interacts with PP2A to facilitate the dephosphorylation of conventional PKC (cPKC), which can be abolished by FADD deficiency or phosphorylation [21]. It has been suggested that overexpression of PKC-α inhibited insulin-induced Akt phosphorylation and activation [31]. Meanwhile, previous work showed that mRNA level of PKC-α in thymocytes was down-regulated by PMA and ionomycin treatment [32], indicating that accumulation of PKC-α may be an inhibitor of thymocytes activation. Thus, we speculate that increased stability and activity of PKC-α resulting from FADD deficiency may block the signal transduction from the over-activated Akt to the downstream mTOR and cause decreased glucose uptake in thymocytes (Fig. 4). In subsequent studies, we will put emphasis on investigating the concrete regulation mechanisms in glucose metabolism mediated by FADD and PKC-Akt pathway in early T cell development.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.092.

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