Marked Reduction in FoxO1 Protein by its Enhanced Proteasomal Degradation in *Zfat*-deficient Peripheral T-Cells

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Abstract. *Background: Zfat is a nuclear protein that harbours putative DNA-binding domains. T-cell specific deletion of Zfat in Zfat$^{f/f}$-CD4Cre mice yields a significant decrease in the number of peripheral T-cells with a lower surface expression of interleukin-7 receptor-α (IL-7Rα). However, the molecular mechanism by which Zfat controls IL-7Rα expression remains unknown. Materials and Methods: Expression levels of the molecules involved in IL-7Rα expression were determined by immunoblotting. Results: Zfat-deficient peripheral T-cells showed a marked reduction in the FoxO1 protein that regulates IL-7Rα expression; however, the FoxO1 mRNA expression level was not affected by Zfat-deficiency. Furthermore, treatment of Zfat-deficient T-cells with a proteasome inhibitor, epoxomicin, restored FoxO1 expression levels, indicating that the loss of Zfat enhanced the proteasomal degradation of the FoxO1 protein. Conclusion: These results suggest that Zfat is required for peripheral T-cell homeostasis through IL-7Rα expression by controlling the FoxO1 protein.*

Zfat (zinc-finger protein in autoimmune thyroid disease susceptibility region/zinc-finger protein with AT-hook) is a nuclear protein that harbours an AT-hook domain and 18-repeats of the C2H2 zinc-finger motif of which amino acid sequences are highly conserved from fishes to higher primates (1, 2). Zfat plays critical roles in early embryonic development, cell survival and cellular differentiation (3-7).

Zfat was originally identified as a candidate susceptibility gene for autoimmune thyroid disease (2). In fact, Zfat is highly expressed in T- and B-cells in immune-related tissues (1, 8). T-cell-specific deletion of the *Zfat* gene in mice by crossing *Zfat*$^{f/f}$ mice with *LckCre* mice yields a drastic reduction in the number of $CD4^+CD8^+$ double-positive (DP) thymocytes (9). *Zfat*-deficiency in DP thymocytes results in de-regulated activations of ERK, JNK and p38 MAPKs implying that Zfat is an essential molecule for the development and survival of the thymic T-cells probably through regulation of MAPK signaling pathway (9, 10). Furthermore, *Zfat*$^{f/f}$*-CD4Cre* mice showed a significant reduction in the number of both $CD4^+$ and $CD8^+$ T-cells in the peripheral immune tissues (11). *Zfat*-deficient peripheral T-cells exhibited a severe defect in proliferation with an impaired induction of interleukin-2 receptor-α (IL-2Rα) in response to T-cell receptor (TCR) stimulation (11). Furthermore, *Zfat*-deficient peripheral T-cells showed a reduced surface expression level of interleukin-7 receptor α (IL-7Rα) (11). Interleukin-7 (IL-7) is a cytokine, which plays a pivotal role in the modulation of the homeostasis of peripheral T-cells (12, 13). The control of IL-7 signaling is mainly dependent on the surface expression level of IL-7Rα (14). Therefore, Zfat plays a critical role in the maintenance of peripheral T-cell homeostasis through regulation of IL-7Rα expression. However, the molecular mechanism by which Zfat controls IL-7Rα expression remains unknown.

FoxO1 is a transcription factor of the forkhead box O (FoxO) family that regulates many cellular processes, including cell-cycle regulation, cell survival and metabolism (15). FoxO1 activity is stringently regulated by nutrients, growth factors and cytokines. In response to these signals, particular kinases, downstream of phosphatidylinositol-3 kinase phosphorylate FoxO1, result in the translocation of

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Key Words: Zfat, FoxO1, proteasomal degradation, IL-7Rα, T-cell homeostasis.

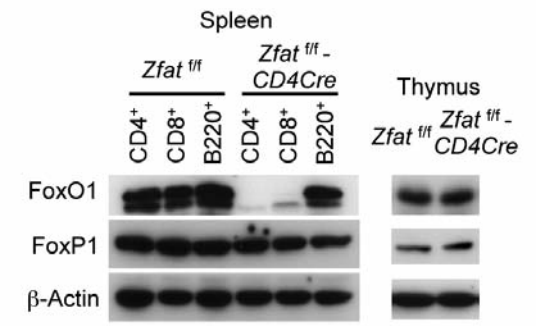

Figure 1. *Marked reduction in the FoxO1 protein levels in the splenic CD4+ and CD8+ T-cells in Zfat$^{f/f}$-CD4Cre mice. Immunoblot of FoxO1 and FoxP1 on the splenic CD4+ T-cells, CD8+ T-cells and B cells (left panel) and whole thymus (right panel) from Zfat$^{f/f}$ and Zfat$^{f/f}$-CD4Cre mice. β-Actin was used as a loading control. Data are representative of three independent experiments.*

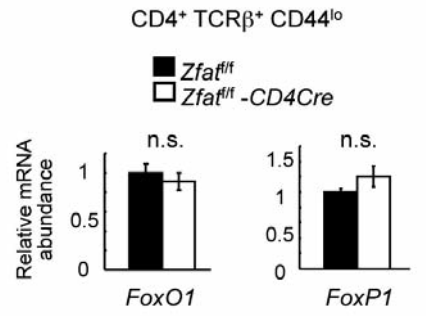

Figure 2. *mRNA expressions of FoxO1 and FoxP1 are not changed in Zfat-deficient CD4+ T-cells. Quantitative RT-PCR analysis of FoxO1 and FoxP1 mRNA expression in the splenic CD4+TCRβ+CD44lo-naive CD4+ T-cells from Zfat$^{f/f}$ (black bars, n=6) and Zfat$^{f/f}$-CD4Cre (white bars, n=9) mice. The relative expression for each gene was normalized by expression of β-actin (Actb). The data are the mean±standard deviation of three independent experiments. n.s., Not significant.*

FoxO1 from nucleus to cytosol and subsequent to its proteasomal degradation (16). Several recent studies have shown an importance of FoxO1 in the immune system, including peripheral T-cell homeostasis (17). Indeed, FoxO1 controls *IL-7Rα* mRNA expression by binding to the regulatory region of the *Il7ra* gene in T-cells (18, 19).

Herein, we showed that *Zfat*-deficiency in the peripheral T-cell results in a drastic decrease in *FoxO1* protein expression. Surprisingly, the *FoxO1* mRNA level was not affected in the *Zfat*$^{f/f}$-CD4Cre T-cells. Treatment with a proteasome inhibitor restored the FoxO1 protein level in the *Zfat*$^{f/f}$*-CD4Cre* T-cells suggesting a deregulated proteasomal degradation of FoxO1 in the *Zfat*-deficient T-cells. These data imply that Zfat is required for the peripheral T-cell homeostasis through IL-7Rα expression by controlling FoxO1 protein.

Materials and Methods

Antibodies. The following antibodies were purchased from BD Biosciences (San Jose, CA, USA): CD4 (RM4-5), CD8 (53-6.7), B220 (RA3-6B2), CD44 (IM7) and TCRβ (H57-597). The antibodies used were as follows: anti-actin (A2066) from Sigma-Aldrich (St. Louis, MO, USA), anti-FoxO1 (C29H4) and anti-Akt from Cell Signaling (Danvers, MA, USA), anti-Skp2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-FoxP1 from EPITOMICS (Cambridge, MA, USA) and anti-Mdm2 from Calbiochem (Billerica, MA, USA). Anti-Zfat antibody was prepared as described previously (1).

Flow cytometry. Cells from spleen and thymus (C57BL/6; CLEA, Tokyo Japan) were depleted of erythrocytes by hypotonic lysis. Cells were incubated with specific antibodies in the presence of 2.4G2 antibody (anti-Fcγ receptor; BD Biosciences). Data were collected with a cytometer (FACSAria II; BD Biosciences) and were analysed with the FlowJo software (FLOWJO, Ashland, OR, USA). Cell separation was performed with MACS Cell Separation Reagents, CD4 MicroBeads, CD8 MicroBeads and B220 (CD45R) MicroBeads (Miltenyi, San Diego, CA, USA) or by using the cell sorting function of the FACSAria II.

Quantitative real-time polymerase chain reaction (RT-PCR). Total RNA was extracted from separated cells using a Qiagen RNeasy kit (Qiagen, Venio, Netherlands). Quantitative real-time polymerase chain reaction was performed using a LightCycler FastStart DNA Master SYBR Green I (Roche, South San Francisco, CA, USA) and ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Waltham, MA, USA) as described previously (1, 7). The PCR primers used for each gene were *FoxO1*, 5'-CATTCCGTCCATCGGTGTTTC-3' and 5'-TCTCATGCAAACCAGGCCTTC-3'; *FoxP1*, 5'-CATGAACCCGC ATGCCTCTAC-3' and 5'-ACAGCCTGGCCACTTGCATAC-3'; and β-Actin (*Actb*), 5'-CATCCGTAAAGACCTCTATGCCAAC-3' and 5'-ATGGAGCC ACCGATCCACA-3'.

Immunoblotting. Separated cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche) and subjected to immunoblotting, as described previously (1). The quantitative analysis of the immunoblots was performed using the integration value of each blot through the measurement module (BZ-H1M; Keyence, Osaka, Japan). β-Actin intensity was used as a loading control and the relative intensity of the signal was normalised by the signal-intensity of protein expression in the cellular lysate of *Zfat*$^{f/f}$ mice as 1.

Statistical analysis. The data were presented as the means±standard deviation (SD). The statistical analyses were performed using an unpaired two-tailed Student's *t*-test. Differences with $p<0.05$ were considered statistically significant.

Results

Marked reduction in the FoxO1 protein levels in the splenic CD4+ and CD8+ T-cells in Zfat$^{f/f}$-CD4Cre mice. To address the molecular mechanisms by which Zfat-deficiency causes a

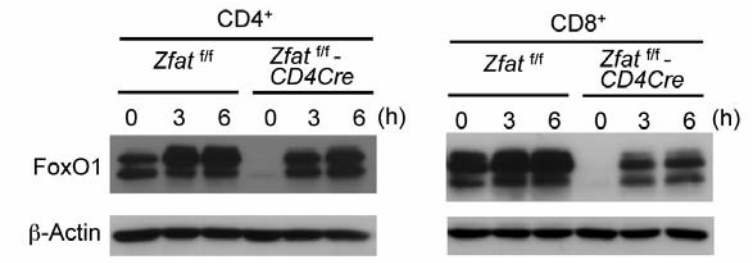

Figure 3. *Restoration of FoxO1 protein level by a proteasome inhibitor. Immunoblot of FoxO1 on the splenic CD4+ (left) and CD8+ T (right)-cells from Zfatf/f and Zfatf/f-CD4Cre mice after treatment with a proteasome inhibitor, epoxomicin. β-Actin was used as a loading control. Data are representative of three independent experiments.*

reduced surface expression of IL-7Rα, we first examined the expression levels of FoxO1 and FoxP1, both of which control the expression of *Il7rα* mRNA in T-cells (18-20). Remarkably, the expression level of the FoxO1 protein, but not that of the FoxP1 protein, was dramatically decreased in splenic $CD4^+$ and $CD8^+$ T-cells; however, this was not the for case B-cells in *Zfat*$^{f/f}$-CD4Cre mice compared with those in *Zfat*$^{f/f}$ mice. By contrast, the levels of FoxO1 and FoxP1 protein expression in the thymus were comparable between genotypes (Figure 1A). However, neither the expression of *FoxO1* nor *FoxP1* mRNA was affected by *Zfat*-deficiency in naive $CD4^+$ T-cells (Figure 2). These data indicate that *Zfat*-deficiency caused a marked reduction in FoxO1 protein levels in splenic $CD4^+$ and $CD8^+$ T-cells without affecting its mRNA expression; this suggested that a decreased expression of IL-7Rα in *Zfat*-deficient peripheral T-cells may result from de-regulation of the FoxO1 protein.

Enhanced proteasomal degradation of FoxO1 in splenic CD4+ and CD8+ T-cells from Zfatf/f-CD4Cre mice. The FoxO1 protein level is mainly regulated through proteasomal degradation (16). Therefore, we next performed an *ex vivo* culture of splenic $CD4^+$ and $CD8^+$ T-cells in the presence of a proteasome inhibitor, epoxomicin. Treatment of T-cells with epoxomicin drastically restored the FoxO1 protein levels in the $CD4^+$ and $CD8^+$ T-cells from *Zfat*$^{f/f}$-CD4Cre spleen (Figure 3). Indeed, the FoxO1 protein levels in the $CD4^+$ T-cells from *Zfat*$^{f/f}$-CD4Cre mice that were cultured with epoxomicin for 6 h were comparable to those in the *Zfat*$^{f/f}$ $CD4^+$ T-cells (Figure 3). These results indicated that the proteasomal degradation of FoxO1 was enhanced in the peripheral T-cells from *Zfat*$^{f/f}$-CD4Cre mice.

Expression of molecules involved in FoxO1 degradation in Zfat-deficient T-cells. Proteasomal degradation of FoxO1 requires the ubiquitination by SCF E3 ubiquitin ligase complex containing Skp2 following Akt-mediated phosphorylation (21). Therefore, we examined the effect of *Zfat*-deficiency on the expression of Skp2 and Akt. However, analyses of Skp2 and Akt levels did not reveal any differences

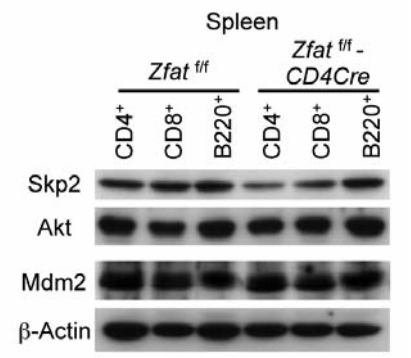

Figure 4. *Zfat-deficiency does not affect the expression levels of Skp2, Akt or Mdm2 protein. Immunoblot of Skp2, Akt and Mdm2 on the splenic CD4+, CD8+ T-cells and B-cells from Zfatf/f and Zfatf/f-CD4Cre mice. β-Actin was used as a loading control. Data are representative of three independent experiments.*

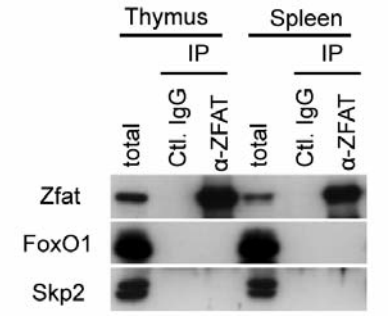

Figure 5. *No physical interaction of Zfat with FoxO1 or Skp2. Anti-Zfat or control (Ctl.) IgG immunoprecipitations were performed using the cell lysates from the thymus or spleen, followed by immunoblotting with anti-Zfat, anti-FoxO1 or anti-Skp2 antibody.*

between *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$*-CD4Cre* T-cells (Figure 4). Mdm2 is also known to be an E3 ubiquitin ligase for FoxO1 (22). However, the expression level of the Mdm2 protein was comparable between the genotypes (Figure 4). Finally, we

could not detect any interactions between Zfat and FoxO1 or Skp2 (Figure 5). These results indicated that ubiquitination or phosphorylation was not involved in the enhanced proteasomal degradation of FoxO1 in *Zfat*$^{f/f}$-CD4Cre T-cells. Deregulation of another post-translational modification on FoxO1 protein, such as methylation and acetylation, may cause deregulated proteasomal degradation of FoxO1 in *Zfat*-deficient T-cells.

Discussion

In this study, we showed that the FoxO1 protein was markedly reduced in the peripheral T-cells from *Zfat*$^{f/f}$-CD4Cre mice. *Zfat*-deficiency enhanced the proteasomal degradation of the FoxO1 protein without affecting the transcription of *FoxO1* mRNA. These results indicate that Zfat plays a critical role in controlling FoxO1 protein expression through the regulation of proteasomal degradation in peripheral T-cells.

Recent studies have identified several direct FoxO1-target genes involved in T-cell homeostasis (18, 19). One important target is Il7ra. FoxO1 regulates the expression of *IL-7Rα* by binding to a 3.5 kb upstream region of the transcription start site of the *Il7ra* gene (19). Indeed, deletion of *FoxO1* gene in mice causes the loss of IL-7Rα in the peripheral T-cells (18, 19). Given that IL-7Rα expression is decreased in *Zfat*-deficient peripheral T-cells (11), Zfat is an important molecule for IL-7Rα expression by controlling FoxO1 protein levels in the peripheral T-cells. Furthermore, FoxO1 also directly regulates the expression of the Klf2 transcription factor, which in turn controls the expression of L-selectin and C-C chemokine receptor type 7 (CCR7) (18, 19). These receptors are important for T-cell homing. Interestingly, the expression of both L-selectin and CCR7 was decreased in *Zfat*-deficient naive CD4$^+$ T-cells from lymph nodes (11). Therefore, Zfat is also involved in the regulation of peripheral T-cell homing through regulation of FoxO1. Taken together, Zfat may link survival and homing of the peripheral T-cells by regulating IL-7Rα, L-selectin and CCR7 by controlling FoxO1 protein expression.

In conclusion, we demonstrated that *Zfat*-deficiency in the peripheral T-cells results in a marked reduction of FoxO1 protein levels due to its enhanced proteasomal degradation, thus indicating that Zfat is required for the proper maintenance of peripheral T-cell homeostasis by controlling FoxO1 protein expression. Therefore, elucidation of the precise molecular mechanisms by which Zfat regulates FoxO1 degradation will provide deeper insights into the peripheral T-cell homeostasis.

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