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Deubiquitination and stabilization of T-bet by USP10

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ABSTRACT

The T-box transcriptional factor T-bet is crucial in the development, differentiation and function of Th1 cells. It drives Th1 immune response primarily through promoting expression of Th1 hallmark cytokine IFN-γ. Although T-bet was found associated with many immune-mediated diseases such as asthma and systemic sclerosis, little is known about the regulation of T-bet stability and function. Here we identified USP10, a carboxyl-terminal ubiquitin-processing protease, could interact with T-bet in the nucleus. Overexpression of USP10 directly inhibited T-bet ubiquitination and increased the expression of T-bet. We further confirmed Quercetin, a reported inhibitor of T-bet, could target USP10. Quercetin treatment downregulated USP10 and promoted T-bet degradation in a proteasome dependent way. Moreover, we found USP10 expression was upregulated in asthmatic patient PBMC, suggesting USP10 may maintain high level of T-bet and IFN-γ to fight against Th2-dominated inflammation.

1. Introduction

T-bet belongs to the T box family of transcriptional factor, controlling the Th1 genetic program in naïve $CD4^+$ T cells [1]. It expresses in several different cell lineages of the hematopoietic system, including DCs, NK cells, NKT cells, B cells and $CD8^+$T cells, and has important roles in the pathogenesis of several autoimmune diseases [2,3]. In the colitis model, a rapid increase of T-bet is showed in the gut mucosal immune system [4]. T-bet expression in DCs is required for the initiation of autoimmune type I diabetes [5]. $Tbx21^{-/-}$ mice exhibit more severe airway hyperresponsiveness and airway remodeling following allergen sensitization and challenge, similar to human chronic asthma [6–8]. Thus it allows of no delay to focus on T-bet and probe further into its function in different diseases.

T-bet could bind to a highly conserved T-box half-site in the IFN-γ promoter and lead to the methylation of the promoter [9]. C-Abl-mediated tyrosine phosphorylation of the T-bet DNA-binding domain was found regulating $CD4^+$ T-cell differentiation and confirmed in allergic lung inflammation [10]. Lys-313 was lately found as a key site required for T-bet to interact with the IFN-γ gene promoter and to assure phosphorylation at Thr-302, thereby suppressing NFAT1 activity [11]. They also found that T-bet expression is controlled by the ubiquitin-proteasomal degradation pathway on the site of Lys-313 [11]. However, the mechanism underlying T-bet regulation by ubiquitination modification remains unclear.

We investigated the role of deubiquitinases involved in T-bet stability and function. Our findings showed that USP10 could stabilize T-bet via deubiquitination, and enhance the secretion of IFN-γ. USP10 was further identified as a target of Quercetin. This suggested the possible mechanism in which Quercetin treatment could inhibit both T-bet and IFN-γ in Th1 cells. The expression level of USP10 is also associated with inflammation. USP10 was highly upregulated in PBMC from asthmatic patients. Altogether, our study described a positive regulator of T-bet and revealed an unknown mechanism modulating T-bet protein expression level that ultimately affects the inflammatory process, with implications for a variety of diseases resulting from hyper-inflammation responses.

2. Materials and methods

2.1. Reagents

The antibodies we used were as follows: anti-Flag (M2, Sigma), anti-Myc (9E10, Santa Cruz), anti-HA (F-7, Santa Cruz), anti-β-actin (AC-74, Sigma), anti-GAPDH (1C4, Sungene Biotech), anti-T-bet (4B10, eBioscience) and anti-USP10 (2E1, Abcam) CHX and MG132 was purchased from Merck. Protein AG-beads were obtained from

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Santa Cruz. Quercetin dehydrate powder was purchased from Shanghai Sangon Biotech and dissolved in Propylene glycol.

2.2. Plasmids

The Plasmids pIPHA-T-bet, pIPMyc-USP10, pFLAG-Ubi and β-gal were maintained in our lab. USP10 C424A mutant was made using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's standard procedure, and confirmed by DNA sequencing. Primers were designed on PrimerX software. OC2-Luc was amplified from Human genomic DNA and cloned into pGL3-Basic reporter vector (Promega). The cloning primers are: 5′-cctccacagccactggtagcttc-3′ and 5′-gtgcccagactttccattgtcagctc-3′.

2.3. Cell culture and transfection

HEK293T cells and Hela cells were cultured in Dulbecco's modified Eagle's medium(DMEM, Hyclone, Thermo) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and streptomycin at 37 °C in the presence of 5% CO_2. The cells were transfected with the indicated plasmids using Polyethylenimine (PEI, Sigma) according to the manufacturer's instructions.

2.4. Immunoblot analysis and immunoprecipitation

Cells were lysed in RIPA buffer containing 50 mM Tris/HCl, pH 7.4, 1% Nonidet P-40, 0.5% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, with 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitor (Sigma), followed by immunoprecipitation with the indicated antibodies, separation by SDS/PAGE, and analysis by Western blotting. The blotting exposure was developed with LAS-4000 Mini system. Where applicable, band density indicating protein amount was quantified using Image J software.

2.5. Human $CD4^+$ T cells isolation

Human PBMCs were isolated by Ficoll (GE) from the buffy coat of healthy donors (Shanghai Blood Center). Human $CD4^+$ T cells were purified using a FACS ARIA II cell sorter (BD). The purity of the sorted cells was 95–99%. The purified human $CD4^+$ T cells were expanded with anti-CD3/CD28 beads (Invitrogen) in X-Vivo media (Invitrogen) with 10% AB serum (Gibco).

2.6. Luciferase reporter assay

The OC2 luciferase reporter plasmid was co-transfected with a β-gal or other target plasmids as indicated into 293T. The cells were lysed and analyzed using a luciferase assay normalized to β-gal activity according to the manufacturer's protocol (Beyotime). Results presented are the mean of three separate experiments, and the error bars indicate standard deviations from the mean.

2.7. Quantitative real-time PCR

Total RNA was extracted from $CD4^+$ T cells with TRIzol reagent (Invitrogen) following the manufacturer's instructions. RNA was quantified and complementary DNA was reverse-transcribed using PrimeScript® RT reagent Kit (Takara) following the manufacturer's instructions. PCR reactions for detecting human genes were carried out using SYBR green mix (TAKARA) on ABI Prism 7900 Sequence Detection System. Quantification of relative mRNA expression was determined by the formula 2-ΔCT normalized to GAPDH expression. The primers for real-time PCR were listed as follows: *Tbx21*-forward: 5′-cacctgttgtggtccaagttt-3′ and *Tbx21*-reverse: 5′-tgacaggaatgggaacatcc-3′; *FOXP3*-forward: 5′-tcccagagttcctccacaac-3′ and *FOXP3*-reverse: 5′-attgagtgtccgctgcttct-3′; *GATA3*-forward: 5′-ctcattaagccccaagcgaag-3′ and *GATA3*-reverse: 5′-ttttcggtttctggtctgg-3′; *USP10*-forward: 5′-tgcagagttgctggagaatg-3′ and *USP10*-reverse: 5′-ggccttt gcactttggaata-3′; *IFN-γ*-forward: 5′-aacgagatgacttcgaaaagc-3′ and *IFN-γ*-reverse: 5′-atattgcaggcaggacaacc-3′; *GAPDH*-forward: 5′-gagt caacggatttggtcgt-3′ and *GAPDH*-reverse: 5′-gacaagcttcccgttctcag-3′.

2.8. Immunofluorescence

The transfected cells were fixed in 4% formaldehyde, permeabilized in 0.5% Triton-X 100, blocked, and incubated with indicated antibody. Cell nuclei were stained with DAPI dye. And slides were imaged on a laser microscope (LEICA SP5).

2.9. Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was carried out using Prism software (Graphpad Software). Comparisons between two groups were done by using the Student t test (unpaired, two-tailed). $P < 0.05$ with a 95% confidence interval was considered significant. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results

3.1. USP10 interacts with T-bet

Several reports have shown that T-bet is regulated by ubiquitination modification and is involved in a proteasome dependent degradation [12,13]. To find out key factors regulating T-bet ubiquitination, we analyzed any possible deubiquitinases (DUBs) interacting T-bet from our pool containing the DUBs highly expressed in T cells. We found the obvious association between T-bet and USP10 in our screening. The physical interaction was demonstrated by endogenous co-immunoprecipitation (Co-IP). USP10 was detected by pull-down with anti-T-bet antibody in human primary $CD4^+$ T cells (Fig. 1A).

Since T-bet is a transcription factor, it mainly localizes in the nucleus, while USP10 is found in both cytoplasm and nucleus [14,15]. We followed up Immunofluorescence after transfecting HA-T-bet and Myc-USP10 into Hela cells and found that the majority of USP10 expressed in the nucleus and colocalized with T-bet (Fig. 1B). The interaction and colocalization suggested USP10 is a binding partner of T-bet.

3.2. USP10 stabilizes T-bet protein via deubiquitination

As a transcription factor, T-bet could bind to the promoter of several genes such as IFN-γ and ONECUT (OC2) and regulate their transcription [16]. To test whether USP10 could affect T-bet function, we constructed a luciferase report gene under the control of OC2 promoter (OC2-Luc). The luciferase activity was enhanced when overexpression of T-bet. The cotransfection of USP10 could significantly upregulated T-bet mediated transcription activation (Fig. 2A), suggesting that USP10 may act as a positive regulator of T-bet.

We further wondered whether USP10 could affect T-bet protein stability. Then we treated cells with cycloheximide (CHX), the protein biosynthesis inhibitor, to measure the half-life of T-bet protein. In the presence of CHX, T-bet was reduced at the protein level; however, the administration of proteasome inhibitor MG132 prevented T-bet degradation in CHX treatment, which is consistent with the previous reports that T-bet undergoes a proteasome dependent pathway (Fig. 2B). When cotransfected with USP10, T-bet degradation by CHX was inhibited (Fig. 2B), demonstrating USP10 stabilized T-bet protein and prolonged the half-life of T-bet.