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Ubiquitination of ROR γ t at Lysine 446 Limits Th17 Differentiation by Controlling Co-activator Recruitment

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Abstract

The transcription factor retinoid acid-related orphan receptor gamma t (ROR γ t) directs the differentiation of T helper 17 (Th17) cells. Th17 cells mediate pathological immune responses responsible for autoimmune diseases, including psoriasis and multiple sclerosis. Previous studies have focused on ROR γ t target genes and their function in Th17 differentiation. Here, we studied post-transcriptional regulation of ROR γ t and identified a functional ubiquitination site, lysine 446 (K446). Mutation of K446 to arginine (K446R) to prevent ubiquitination greatly enhanced recruitment of steroid receptor co-activator 1 (SRC1), a co-activator critical for ROR γ t activity. Correspondingly, the K446R mutation potentiated Th17 differentiation. We also showed that the ubiquitin specific protease 15 (USP15) interacted with ROR γ t, removed ubiquitin from K446, and stimulated ROR γ t activity by enhancing co-activator SRC1 recruitment. Knockdown of USP15 or expression of inactive USP15 impaired Th17 differentiation, suggesting a positive role for USP15-mediated deubiquitination of ROR γ t in Th17 differentiation. Therefore, ubiquitination of K446 limits ROR γ t-mediated Th17 differentiation by inhibiting the recruitment of co-activator SRC1. Our study will inform the development of treatments that target ROR γ t ubiquitination pathways to limit Th17-mediated autoimmunity.

Introduction

CD4⁺-derived T helper 17 (Th17) cells contribute to protective immunity against pathogens (1–3), as well as pathological immune responses responsible for autoimmune diseases, including psoriasis and multiple sclerosis (4, 5). Retinoid acid-related orphan receptor gamma t (ROR γ t) is the well-known transcription factor that directs Th17 differentiation (6,

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7). Activation of naïve T cells in the presence of TGF β and IL-6 is sufficient for up-regulation of ROR γ t and subsequent differentiation into Th17 cells (8, 9). Addition of IL-23 and IL-1 further potentiates Th17 differentiation (10–12). Mutation of the ROR γ t gene causes severe immunodeficiency in both mice (6) and humans (13). Because it is a transcription factor, most previous studies have focused on ROR γ t target genes critical for the regulation of Th17 differentiation, and have demonstrated that ROR γ t directly stimulates the expression of genes, including IL-17 (6, 7, 14, 15). However, little is known about the mechanisms responsible for post-transcriptional regulation of ROR γ t. Because Th17 cells produce the effector cytokines including IL-17A, IL-17F, IL-22, and GM-CSF to mediate pathological inflammation (16–18), targeting Th17 cells is considered a potential treatment for autoimmune disease (19). Pharmaceutical and academic scientists have developed ROR γ t inhibitors for treatment of Th17-dependent autoimmunity (20–24). Therefore, understanding the post-transcriptional regulation of ROR γ t will not only reveal the basic mechanisms for regulating Th17 differentiation, but may also facilitate the development of novel therapeutics for the treatment of autoimmunity.

ROR γ t is a member of the steroid nuclear receptor superfamily and consists of three domains (25, 26): 1) a conserved DNA-binding domain with two zinc finger motifs responsible for DNA-binding; 2) a conserved ligand-binding domain with a carboxy-terminal activation function 2 (AF2) motif responsible for recruiting steroid receptor co-activators (SRC) such as SRC1 (27, 28); and 3) a hinge domain in between the DNA-binding and ligand-binding domains. Upon ligand binding, ROR γ t binds to target DNA and recruits SRC1 that associate with histone acetyltransferases and methyltransferases to stimulate target gene expression (29). Regulation of co-activator recruitment is an important step for controlling ROR γ t activity; mutation within the AF2 motif that prevents the recruitment of SRC1 abrogates ROR γ t gene-stimulating activity (28, 30). Furthermore, pharmacological ROR γ t inhibitors developed to date inhibit ROR γ t activity by interfering with the interaction between ROR γ t and SRC1 (21, 30, 31).

Ubiquitination is a post-transcriptional modification that regulates many aspects of cellular function (32). Ubiquitin ligase enzymes catalyze the addition of ubiquitin to lysines of the protein substrates, whereas deubiquitinases are responsible for removing ubiquitin. There are five families of deubiquitinases, and ubiquitin specific proteases (USPs) are the largest of these families (33). One of the functions of ubiquitination is to regulate protein stability by inducing protein degradation. Evidence indicates that ROR γ t stability can be regulated by ubiquitination (34–37). Another important role for ubiquitination is to regulate protein-protein interactions. It remains unknown whether ubiquitination plays a role in regulating the interactions between ROR γ t and its associated proteins in Th17 differentiation. In this study, we identified a ubiquitination site, ROR γ t K446, that negatively regulates Th17 differentiation by controlling the interaction between ROR γ t and SRC1. Mutation of K446 to arginine (K446R) stimulates ROR γ t activity by enhancing ROR γ t-SRC1 interaction. Correspondingly, K446R is more potent than wild-type (WT) ROR γ t in rescuing Th17 differentiation when expressed in *ROR γ t^{-/-}* T cells. Furthermore, ubiquitin specific protease 15 (USP15) binds ROR γ t to deubiquitinate K446, leading to stimulation of ROR γ t activity by promoting the ROR γ t-SRC1 interaction. Moreover, knockdown of UPS15, or expression of inactive USP15, impaired Th17 differentiation. We thus identified a ROR γ t ubiquitination

site, K446, which is deubiquitinated by USP15 to control ROR γ t interaction with co-activator SRC1 and ultimately regulates Th17 differentiation.

Materials & Methods

Mice

Wild-type (WT) and *ROR γ t*^{-/-} C57BL/6 mice have been described previously (38). Mouse care and experimental procedures were performed under specific pathogen-free conditions following institutional guidance and approved protocols from the Institutional Animal Care and Use Committee at the Beckman Research Institute, City of Hope (no. 07023).

Plasmids and antibodies

A retroviral expression plasmid [murine stem cell virus (MSCV)-IRES-GFP] was used to clone *ROR γ t* (*Mus musculus*, NM_001293734.1) or SRC1 (*Mus musculus*, NM_010881.2) cDNA. Specific point mutations of ROR γ t were introduced by using a mutagenesis kit (Agilent Technologies, CA, USA). LMP vector-based retroviral USP15 (*Mus musculus*, NM_027604.4) and USP45 (*Mus musculus*, NM_152825.2) short hairpin RNA (shRNA)-expressing vectors (LMP-USP15 shRNA1 and shRNA2; LMP-USP45 shRNA1 and shRNA2) were constructed by using following oligonucleotide sequences: shUSP15-1, TGCTGTTGACAGTGAGCGACCAGACTGTGGAACAAGTATATAGTGAAGCCACAGATGTATA TACTTGTTCCACAGTCTGGCTGCCTACTGCCTCGGA; and shUSP15-2, TGCTGTTGACAGTGAGCGCGGACAGGTGTTAGTGATAGAATAGTGAAGCCACAGATGTATT CTATCACTAACACCTGTCCTTGCCTACTGCCTCGGA. shUSP45-1, TGCTGTTGACAGTGAGCGCCACTTTCTCCTAAAGTTCCTTAGTGAAGCCACAGATGTAAAG AACTTTAGGAGAAAGTGGTTGCCTACTGCCTCGGA and shUSP45-2, TGCTGTTGACAGTGAGCGAGCATCTTTATTGGCGAATTAATAGTGAAGCCACAGATGTATTAATTCGCCAATAAAGATGCGTGCCTACTGCCTCGGA. USP15 was amplified from pCMV6-Kan/Neo-USP15 (Origene Technology Inc, MD, USA) and inserted into pMSCV-IRES-GFP. USP15 cysteine 298 was mutated to alanine using a mutagenesis kit (Agilent Technologies, CA, USA). Hamster anti-ROR γ antibody was purchased from Biolegend (Biolegend, CA, USA). Rabbit anti-USP15 antibody was purchased from Bethyl Laboratories, Inc (Bethyl Laboratories, TX, USA). Mouse anti-SRC1 antibody, mouse anti-Actin antibody, mouse anti-HA antibody and mouse anti-FLAG antibody were purchased from Sigma-Aldrich (Sigma-Aldrich Inc., MO, USA). Rabbit anti-GFP antibody was purchased from Life Technologies (Life Technologies Inc., CA, USA).

Cell culture, transient transfection and reporter assay

EL-4 cells were cultured in RPMI1640 supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. 293T cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells (2×10^5 in each well of a 24 well-plate) were transfected with the reporter plasmid (100 ng), pSV40-*Renilla* luciferase vector (50 ng), and expression vectors (0.5 μ g) by using BioT (Bioland Scientific LLC, CA, USA). The total amount of transfected DNA was kept constant by adjusting the amount of the empty vector. Cells were collected after 24 h and lysed in 200 μ l lysis buffer (137 mM NaCl, 50 mM Tris-HCl, 0.5% NP-40);

luciferase activities were measured by the Dual Luciferase system, according to the manufacturer's instructions (Promega, WI, USA), and normalized against *Renilla* luciferase activities. "Folds of stimulation" represents normalized luciferase activity divided by the result of reporter-only groups.

T cell isolation and in vitro Th17 differentiation

CD4⁺ T cells were isolated from spleens of 6- to 10-wk-old C57BL/6 mice by negative selection using a CD4⁺ T cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) and replicate suspensions of 4×10^5 /ml iDMEM with 10% FBS, 100 U/ml penicillin G, and 50 µg/ml streptomycin were cultured in 24-well plates that had been precoated with 0.2 mg/ml goat anti-hamster Ab. The medium was supplemented with 0.25 µg/ml hamster anti-CD3 (145-2C11; eBioscience, CA, USA), 1 µg/ml hamster anti-CD28 (37.51; eBioscience, CA, USA), 2 ng/ml TGF-β1 (eBioscience), and 20 ng/ml IL-6 (eBioscience). T cells were cultured for 3 days at 37°C with 5% CO₂.

Flow cytometry

For intracellular cytokine staining, cells obtained from *in vitro* cultures were incubated for 4–5 h with 50 ng/ml PMA, 750 ng/ml Ionomycin (both Sigma-Aldrich Inc., MO, USA) and 10 µg/ml Brefeldin A (BD) in a tissue culture incubator at 37°C. Cells were resuspended in Fixation/Permeabilization solution (BD Cytofix/Cytoperm Kit, BD Pharmingen, USA), and intracellular cytokine staining was performed according to the manufacturer's protocol. Cells were intracellularly stained with anti-mouse IL17 (TC11-18H10.1) and analyzed using a BD FACSCanto II flow cytometer (BD Biosciences, CA, USA) and FlowJo software (Tree Star, OR, USA).

Retroviral packaging and transduction

Retroviral expression plasmids were transfected into Plat-E packaging cells using Lipofectamine 2000 (Invitrogen Life Technologies, CA, USA). After 48 h, viral supernatants were collected, passed through 0.4-µm filters, and stored at –80°C until use. For transduction, naïve T cells were first activated with 0.25 µg/ml hamster anti-CD3 (145-2C11; eBioscience, CA, USA), 1 µg/ml hamster anti-CD28 (37.51; eBioscience, CA, USA) in a 24-well plate precoated with goat anti-hamster Ab for 24 h, then spin-infected with viral supernatants (2500 rpm, 30°C for 2 h) in the presence of 8 µg/ml polybrene (Sigma-Aldrich, MO, USA). After spin infection, indicated cytokines were added to the culture media to induce Th17 differentiation.

RT-PCR and quantitative PCR

Total RNA was prepared using TRIzol (Invitrogen Life Technologies, CA, USA), and cDNA was prepared by using Tetro cDNA synthesis kit (Bioline Inc, London, UK). Primers for RT-PCR (5' to 3') were as follows: IL17A Forward: TTAACTCCCTTGCGCAAAA, IL17A Reverse: CTTTCCCTCCGCATTGACAC; IL17F Forward: TGCTACTGTTGATGTTGGGAC, IL17F Reverse: AATGCCCTGGTTTTGGTTGAA; CCR6 Forward: CCTGGGCAACATTATGGTGGT, CCR6 Reverse: CAGAACGGTAGGGTGAGGACA; CCL20 Forward: GCCTCTCGTACATACAGACGC,

CCL20 Reverse: CCAGTTCTGCTTTGGATCAGC; IRF4 Forward: TCCGACAGTGGTTGATCGAC, IRF4 Reverse: CCTCACGATTGTAGTCCTGCTT; ROR α Forward: GTGGAGACAAATCGTCAGGAAT, ROR α Reverse: TGGTCCGATCAATCAAACAGTTC; AHR Forward: AGCCGGTGCAGAAAACAGTAA, AHR Reverse: AGGCGGTCTAACTCTGTGTTC; Beta-Actin Forward: GAGTCCTACGACATCATCGCT, Beta-Actin Reverse: CCGACATAGTTTGGGAAACAGT.

Isolation of active deubiquitinases with the HA-VS-Ub Probe

Th17 cells, differentiated *in vitro* as described above, were lysed in 0.1% NP-40, 150 mM NaCl, 20 mM CaCl₂, 50 mM Tris pH 7.4 buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF). Sample preparation was performed as described (39). Briefly, samples containing equal amounts of protein were subjected to enzymatic reaction with the ubiquitin vinyl sulfone HA-tagged probe (Ub-VS-HA; Boston Biochem, USA) at a ratio of ~1:200 for 45 min at 37°C. Treated lysates were separated by SDS-PAGE and analyzed by anti-HA western blotting to visualize the active deubiquitinases. For immunoprecipitation, 8 mg of protein per sample (obtained as described in the section above) was diluted in NET buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, pH 7.4) to a protein concentration of 1 mg/ml, pre-cleared with agarose-coupled Protein A beads (Sigma-Aldrich) for 1 h at 4°C, and immunoprecipitated with 60 μ l of anti-HA Agarose Conjugate (Sigma-Aldrich) for 14 h at 4°C. The resin was washed four times with NET buffer and eluted for 20 min with 0.3 ml of 100 mM glycine pH 2.5.

Sample preparation and mass spectrometric analysis

The immunoprecipitated proteins were eluted and then precipitated using chloroform and methanol: 200 μ l total volume of sample, 600 μ l methanol, and 150 μ l chloroform were vortexed, followed by addition of 450 μ l MilliQ-H₂O and centrifugation at room temperature for 1 min at 14,000 rpm. The upper aqueous phase was discarded. Methanol (450 μ l) was added to the lower phase and interphase, vortexed, and centrifuged at room temperature for 1 min at 14,000 rpm. The supernatants were removed and the pellets were resuspended in 0.1 M Tris pH 7.8 containing 6 M Urea and digested by trypsin digestion. Alternatively, samples were resolved on SDS-PAGE and silver stained using the Pierce Silver Stain for Mass Spectrometry kit (Fisher Scientific), followed by excision of gel bands and tryptic digestion. We then performed on-line 2D nano-LC Q Exactive MS and HCD MS/MS to analyze immunoprecipitated proteins to identify post-transcriptional modification sites of ROR γ t, ROR γ t binding proteins or active DUBs. Especially, to detect the ubiquitination of ROR γ t, immunoprecipitation was first subject to trypsin digestion. Digested ubiquitinated lysine (K) will leave GG isopeptide linked to K, which can be easily detected by MS due to an increased mass of 114.102 dalton. Detection of Ub footprints (GG isopeptide linked to K) at high resolution and a series of fragmentation ions from HCD allowed the accurate sequencing and assignment of the site of modification to only one site. Integrating MS and HCD MS/MS results. Protein Discoverer 1.4 automatically assigned potential modification sites with high confidence.

Enrichment of IL17-producing cells

IL17-producing EL-4 cells were enriched by using a Cell Enrichment and Detection Kit (Miltenyi Biotec Inc, Bergisch-Gladbach, Germany). Briefly, 10^8 cells were harvested and incubated with IL-17 Catch Reagent on ice for 5 min and at 37°C for 45 min under slow continuous rotation. Cells were washed and then stained with IL-17 detection Antibody. IL17-producing cells were sorted by BD FACSAria II cell sorter.

Enrichment of ubiquitinated proteins by Agarose-TUBE2

1.5×10^6 Th17 cells, differentiated *in vitro* as described above, were lysed in 500 μ l cell lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride (PMSF)). Cell lysates were incubated with 20 μ l equilibrated Agarose-TUBE2 (Lifesensors Inc., PA, USA) at 4°C for 4 hours. Agarose-TUBE2 was then washed with 1 ml TBS-T (20 mM Tris-HCl pH8.0, 150 mM NaCl, 0.1% Tween-20) and suspended in SDS reducing sample buffer. Eluted samples were analyzed by western blotting.

Statistical analysis

Statistical analysis was performed using the unpaired, two-tailed Student's t test. A *p*-value <0.05 was considered significant. Flow cytometry as well as immunoblot data shown are representative of at least three independent experiments

Results

K446 of ROR γ t is a ubiquitination site

To study post-transcriptional regulation of ROR γ t, we first determined whether ROR γ t is ubiquitinated. To overcome the difficulty of detecting ubiquitinated proteins, we first used an *in vitro* system to overexpress both ROR γ t and HA-tagged ubiquitin (HA-Ub) in 293T cells (Fig. 1A). A ladder pattern typical of ubiquitinated ROR γ t was detected in the presence, but not absence, of HA-Ub. In T cells differentiated under Th17 conditions (TGF β and IL-6), TUBEs (tandem ubiquitin binding entities) (40) were used to enrich ubiquitinated proteins (Fig. 1B). Ubiquitinated ROR γ t was detected in wild-type (WT) but not ROR γ t^{-/-} cells, clearly suggesting that endogenous ROR γ t is ubiquitinated in Th17 cells. To identify potential ubiquitination site(s), we performed mass spectrometric (MS) analysis of ROR γ t immunoprecipitated from ROR γ t-expressing 293T cells and differentiated Th17 cells treated with deubiquitinase inhibitor. Immunoprecipitated ROR γ t was first digested with trypsin. Digesting ubiquitinated lysine (K) leaves GG isopeptide linked to K, which can be easily detected by MS due to an increased mass of 114.102 daltons (41). In this way, K446 of ROR γ t was identified as an apparent ubiquitination site in both Th17 (Fig.1C) and 293T cells (data not shown) by its MS/MS peptide spectrum. To confirm K446 ubiquitination, we mutated K446 to arginine (K446R) to prevent ubiquitination. Ubiquitination of ROR γ t WT and K446R was then compared in the presence of HA-Ub, but we did not observe obvious differences (data not shown), likely due to presence of other ubiquitination sites. We thus mutated all 24 lysines in ROR γ t to arginines [K(24)R], except K446 (K446) or K433 (K433); K433 was not a ubiquitination site in our MS analysis and was therefore used as a

negative control. As expected, when expression plasmids encoding ROR γ t or mutants and HA-Ub were transfected into 293T cells, no ubiquitination was detected in the K(24)R mutant or K433 control, whereas obvious ubiquitination signals were detected in K446. This confirms that K446 of ROR γ t is a ubiquitination site.

ROR γ t K446 ubiquitination negatively regulates Th17 differentiation

To determine the function of K446 ubiquitination in Th17 differentiation, K446 or K433 (control) were mutated to arginine (K446R and K433R). We developed an assay to evaluate K446R function using retrovirus-reconstituted ROR γ t^{-/-} T cells which cannot differentiate into Th17 cells due to lack of endogenous ROR γ t. This system is a powerful tool, as any Th17 differentiation activity in the reconstituted ROR γ t^{-/-} T cells solely depends on retrovirus-mediated expression of exogenous ROR γ t. T cells obtained from ROR γ t^{-/-} mice (38) were transduced with retrovirus expressing GFP only or together with ROR γ t WT, K446R, or K433R, and differentiated under Th17 differentiation conditions. IL-17+/GFP+ cells were enumerated by flow cytometry (Fig. 2A and 2B), and ROR γ t and mutants expression were monitored (Fig. 2C). Consistent with the role of ROR γ t in Th17 differentiation, ROR γ t^{-/-} T cells expressing ROR γ t differentiated into IL-17 producing cells, but GFP-only control cells did not. Relative to both ROR γ t WT and control mutant K433R, the K446R mutant greatly potentiated Th17 differentiation. We then compared the expression of critical Th17 genes between cells expressing ROR γ t WT and K446R (Fig. 2D). IL-17A, CCR6, CCL20 and ROR α but not IL-17F, IRF4 and Ahr were significantly up-regulated in Th17 cells expressing K446R, confirming the potentiation of Th17 differentiation by K446R. To study Th17 differentiation in re-stimulated cells, the differentiated Th17 cells described above were rested in IL-2 for 48 hrs, and then placed in Th17 priming conditions again (Fig. 2E and 2F). Expression of ROR γ t and mutants were monitored (Fig. 2G). The differences between ROR γ t WT- and K446R-mediated Th17 differentiation (Fig. 2E and Fig. 2F) and expression of critical Th17 genes (Fig. 2H) were further increased. Therefore, our results illustrate that the K446R mutant enhanced Th17 differentiation, demonstrating the negative role of ROR γ t K446 ubiquitination in the regulation of Th17 differentiation.

ROR γ t K446 ubiquitination inhibits ROR γ t activity by preventing the recruitment of co-activator SRC1

An important function of ubiquitination is to regulate protein degradation (32). Reports have suggested a role for ubiquitination in the regulation of ROR γ t stability (34–37). However, in our studies, ROR γ t WT and the K446R mutant expressed at similar levels (Fig. 3A, 3F and 3G). To confirm that K446 ubiquitination does not regulate ROR γ t protein stability, we compared the degradation rates of ROR γ t WT and K446R in the presence of the protein synthesis inhibitor cycloheximide (CHX) (Fig. 3A). The rates were similar, suggesting K446 ubiquitination does not regulate ROR γ t protein stability. Another function of ubiquitination is to regulate protein-protein interactions. We previously identified an activation function two (AF2) motif that is located 33 amino acids downstream of K446 (28). The AF2 motif mediates the recruitment of steroid co-activator one (SRC1), which stimulates ROR γ t-mediated transcriptional activity. Mutation of a critical AF2 tyrosine (479) to phenylalanine (ROR γ t-AF2) disrupted the ROR γ t-SRC1 interaction (Fig. 3B). Expression of ROR γ t-AF2

but not ROR γ t WT (Fig. 3E) failed to rescue Th17 differentiation in *ROR γ t^{-/-}* T cells (Fig. 3C and 3D), strongly supporting the important function of ROR γ t-recruited SRC1 in Th17 differentiation. To determine whether K446 ubiquitination affects ROR γ t-SRC1 interaction, we expressed ROR γ t WT or K446R with SRC1 in 293T cells (Fig. 3F) and *ROR γ t^{-/-}* Th17 cells (Fig. 3G). More SRC1 was immunoprecipitated in cells expressing K446R than WT ROR γ t, suggesting that K446 ubiquitination inhibits SRC1 binding. Transcription activity of ROR γ t depends on the recruitment of co-activator SRC1 (28). To determine the effects of K446R-enhanced SRC1 recruitment on ROR γ t-mediated transactivation, we used an IL-17 promoter-luciferase reporter (Fig. 3H), as ROR γ t directly binds to IL-17 promoter to stimulate its expression (42). In the presence of SRC1, ROR γ t WT and K433R mutant stimulated the IL-17 reporter, consistent with the function of SRC1 as a co-activator for ROR γ t. The K446R mutant activated the IL-17 reporter more stronger than that of the ROR γ t WT. Taken together, our results suggest that K446 ubiquitination inhibits ROR γ t activity by preventing SRC1 recruitment.

USP15 interacts with ROR γ t and deubiquitinates K446

Addition of ubiquitin to lysine residues requires ubiquitin ligase enzymes, whereas removal of ubiquitin is catalyzed by deubiquitinases (32). To determine the enzyme(s) responsible for modifying K446, we used MS to identify ROR γ t-interacting proteins. To obtain the large amount of specific ROR γ t-interacting protein complexes needed for successful MS analysis, we generated a stable EL-4 cell line expressing 3xflag-tagged ROR γ t. EL-4 cells were used because they express ROR γ t and produce IL-17 (43), and thus have the components required for IL-17 production. Flag-tag avoids disguising the epitopes critical for interacting with co-factors and nonspecific binding due to cross-reactivity with anti-ROR γ t antibodies. We confirmed that EL-4 cells produced IL-17 which was further enhanced by overexpressing flag-tagged ROR γ t (Fig. 4A), indicating that flag-tag does not affect ROR γ t function in stimulating IL-17 production. To enrich the protein components critical for regulating IL-17 production, IL-17-producing cells were further enriched to almost 90% purity using IL-17-catching beads (Fig. 4A, right panel). Immunoprecipitation using anti-flag antibody was performed on EL-4 WT and ROR γ t-flag-expressing cell lysates and immunoprecipitated complexes were then resolved on a protein gel (Fig. 4B). As expected, an intense band corresponding to ROR γ t was observed in ROR γ t-flag-expressing, but not WT, EL-4 cells (Fig. 4B). MS analysis of a band observed in ROR γ t-flag-expressing but not WT EL-4 cells revealed a deubiquitinase, ubiquitin specific protease 15 (USP15). ROR γ t-USP15 interaction in the ROR γ t-flag EL4 cells was confirmed by immunoprecipitation analysis (Fig. 4C). We also confirmed the ROR γ t-USP15 interaction in differentiated Th17 cells (Fig. 4D). Interestingly, a catalytically inactive USP15 with a cysteine 298 to alanine mutation (C298A) (44), had reduced interaction with ROR γ t (Fig. 4E), suggesting important function of enzyme activity in ROR γ t-USP15 interaction. To determine whether USP15 is active in Th17 cells, we used a ubiquitin vinyl sulfone HA-tagged probe (Ub-VS-HA) that crosslinks active deubiquitinases (39). Active ubiquitinases were crosslinked to the Ub-VS-HA probe by incubating with differentiated Th17 cell lysates, followed by immunoprecipitation by anti-HA antibody and analyzed by MS (Fig. 4F). Indeed, USP15 was detected in immunoprecipitated active ubiquitinases in differentiated Th17 cells (Fig. 4F) with more than 50% peptide coverage (Fig. 4G). To determine whether USP15 can deubiquitinate

ROR γ t, ROR γ t was co-expressed with USP15 WT or C298A and HA-Ub in 293T cells. WT, but not C298A, USP15 reduced ubiquitination of ROR γ t (Fig. 4H). To determine whether USP15 can deubiquitinate ROR γ t K446 specifically, K446 that has all lysines mutated to arginines except K446 was co-expressed with USP15 WT or C298A and HA-Ub in 293T cells, as we showed that K446 can be ubiquitinated (Fig. 1D). USP15 WT, but not C298A, removed ubiquitin from K446 (Fig. 4I). Taken together, our results demonstrate that USP15 is active and associated with ROR γ t in Th17 cells. More importantly, USP15 is able to deubiquitinate ROR γ t at K446 specifically.

USP15 stimulates ROR γ t activity and Th17 differentiation by enhancing SRC1 recruitment

To determine the role of USP15 in ROR γ t and SRC1-regulated transactivation, we again used the IL-17 promoter reporter. USP15 WT greatly potentiated, whereas inactive C298A rather inhibited, reporter activity (Fig. 5A). Correspondingly, USP15 enhanced (Fig. 5B), whereas inactive C298A inhibited (Fig. 5C), SRC1 recruitment to ROR γ t. This suggests that USP15 stimulates ROR γ t activity via enhancing SRC1 recruitment. Consistent with our previous observation that K446R enhanced SRC1 recruitment (Fig. 5B). Furthermore, K446R-enhanced SRC1 recruitment was independent of the presence of USP15 (Fig. 5B) or C298A (Fig. 5C), strongly suggesting that USP15 regulates SRC1 recruitment via deubiquitinating K446. We next determined whether USP15 is important for Th17 differentiation using short hairpin RNA (shRNA) knockdown. Knockdown of USP15 with two different shRNAs markedly inhibited Th17 differentiation (Fig. 5D, top panels and 5E) compared to knockdown of a close member of USP15, USP45 (Fig. 5D, bottom panels and 5E). shUSP15-2 was more potent in inhibiting Th17 differentiation than shUSP15-1, which correlated well with the greater efficacy of shUSP15-2 to knock down USP15 expression (Fig. 5F). Analysis of critical Th17 genes also confirmed knockdown of USP15 impaired Th17 differentiation (Fig. 5G). In addition, retrovirus expressing USP15 WT or C298A transduced T cells to determine their function in Th17 differentiation. Overexpression of USP15 did not affect Th17 differentiation relative to empty virus expressing GFP alone (EV) (Fig. 5H, 5I and 5J), likely due to already much active USP15 detected in Th17 cells (Fig. 4F). In contrast, the inactive USP15 C298A mutant did inhibit Th17 differentiation. Impaired Th17 differentiation by both inactive C298A and knockdown of USP15 supports the positive role of USP15 in Th17 differentiation. Altogether, our results indicate that USP15 stimulates ROR γ t activity via enhancing SRC1 recruitment, ultimately leading to promoting Th17 differentiation.

Discussion

The generation of robust Th17 immune responses necessary for clearance of pathogens, including fungus and *Citrobacter rodentium*, depends on stimulating the differentiation of Th17 cells (1–3, 45, 46). Conversely, effective prevention of Th17-mediated autoimmune diseases, such as multiple sclerosis and psoriasis, depends on inhibiting Th17 differentiation (4, 17, 18, 21, 47). ROR γ t is the transcription factor that instructs Th17 differentiation (6, 31), making this molecule an attractive drug target for precisely controlling Th17-mediated immunity (22, 24). It is thus important to understand the mechanisms regulating ROR γ t activity. Previous studies have focused on transcriptional regulation of ROR γ t and

identification of ROR γ t target genes (6, 7, 14, 15). Less is known about the mechanisms of post-transcriptional regulation that is responsible for regulating ROR γ t function. Reports that ROR γ t stability is regulated by ubiquitination-mediated degradation mechanisms (34–37) suggested a role for ubiquitination in ROR γ t-mediated differentiation. In this study, we identified ROR γ t K446 as a ubiquitination site that limits Th17 differentiation by interfering with the recruitment of co-activator SRC1. Therefore, ubiquitination of ROR γ t has additional role in the regulation of ROR γ t interaction with its co-factor to regulate Th17 differentiation. This study also reveals the therapeutic potentials for clinically boosting the Th17 immunity against pathogens or preventing pathological Th17-mediated autoimmunity via controlling the ROR γ t ubiquitination pathways.

Like other nuclear receptors, ROR γ t cannot regulate gene transcription by itself; it has to recruit other cofactors such as SRC1 for activation of target genes (29). SRC1 brings in transcriptional regulators, including histone acetyltransferases and methyltransferases, to open the gene locus for active transcription. Recruitment of SRC1 is therefore a critical checkpoint for controlling ROR γ t activity. On the other hand, SRC1 does not directly bind to DNA and depends on transcription factors to bring it to the specific DNA targets on chromatin to regulate gene expression. The fact that SRC1 services as co-activators for many different transcription factors (48) suggests that the transcription factors associated with SRC1 determines the specificity of SRC1 in the regulation of gene expression. Based on the traditional model, ligand binding is considered a critical step for triggering the recruitment of co-activator by nuclear receptors (29). However, ROR γ t ligands, cholesterol intermediate metabolites, are usually abundantly available in cells (30). The question is whether there are other mechanisms regulating ROR γ t to recruit SRC1. We identified K446 of ROR γ t as a ubiquitination site in limiting SRC1 recruitment, and thus suggesting its function in controlling Th17 differentiation. Indeed, prevention of K446 ubiquitination (K446R mutation) promoted Th17 differentiation. Therefore, in addition to transcription regulation of ROR γ t expression and ligand-induced recruitment of SRC1, our results demonstrated additional layer of regulation mechanisms responsible for controlling ROR γ t activity via post-transcriptional ubiquitination. However, it is still not clear how K446 ubiquitination prevents SRC1 binding. Because K446 is close to the critical tyrosine (Y479) in the AF2 motif responsible for recruiting SRC1, it remains to be determined whether K446 ubiquitination physically blocks SRC1 binding. There are several other lysines close to K446 including K433, K442 and K448. Our study does not exclude the possibility that these neighboring lysines can be ubiquitinated and that K446 may even affect ubiquitination of the neighboring lysines. Moreover, the physiological signals regulating K446 ubiquitination are not known. Identification of such signals will facilitate understanding the function of K446 ubiquitination in the regulation of *in vivo* immune responses both in disease and normal conditions.

Another effort in this study was to identify the enzymes responsible for modifying K446 ubiquitination. Since K446 ubiquitination negatively regulates Th17 differentiation, K446 must be deubiquitinated to promote Th17 differentiation under Th17 priming conditions. Our efforts thus focused on identifying the deubiquitinases. Our following strong evidence supports that USP15 deubiquitinates K446 to promote Th17 differentiation by enhancing SRC1 recruitment: 1) USP15 is the active deubiquitinase associated with ROR γ t in Th17

cells and IL-17-producing EL-4 cells; 2) WT, but not inactive (C298A mutation), USP15 is able to remove ubiquitin from K446 of ROR γ t and stimulate ROR γ t-SRC1 interaction and ROR γ t activity; 3) knockdown of USP15 or forced expression of inactive USP15 impaired Th17 differentiation. USP15 has been reported to regulate multiple functions from oncogenesis to bone formation via deubiquitinating and stabilizing protein substrates (49–53), i.e. reversing ubiquitination-induced protein degradation. We report here that USP15 regulates ROR γ t activity via controlling SRC1 recruitment and thus reveal a new mode of action for USP15-regulated function, controlling protein-protein interaction. However, another report has shown that mice with a germline deletion of USP15 did not show defects in Th17 function (49). An obvious explanation for this discrepancy is redundancy, as other deubiquitinases may replace the function of USP15. It is also possible that a germline deletion gives mice enough time during development to adapt to the absence of USP15, for example, through up-regulation of other USPs to compensate for the lack of USP15. In fact, our demonstration that acute knockdown of USP15 affected Th17 differentiation supports the hypothesis that compensation mechanisms are likely developed in USP15 knockout mice. A conditional knockout of USP15 in Th17 cells specifically will facilitate determination of the function of USP15 in Th17 differentiation.

Th17 cells produce effector cytokines IL-17A, IL-17F, IL-22 and GM-CSF to mediate the pathological inflammation responsible for many types of autoimmune diseases; targeting Th17 cells is thus a potentially valuable treatment for these diseases (19). Indeed, inhibiting the Th17 pathway is effective in the treatment of psoriasis and multiple sclerosis (54, 55). For example, ustekinumab, a human monoclonal antibody that inhibits Th17 responses by blocking the IL-23 receptor, has been approved by the FDA for treatment of severe plaque psoriasis (56). Given the essential function of ROR γ t in Th17 cells, pharmaceutical and academic scientists are developing ROR γ t inhibitors for treatment of Th17-dependent autoimmunity (20–24). Such inhibitors are typically screened for the ability to disrupt the interaction between ROR γ t and co-activator SRC1 (20). We demonstrated here that recruitment of SRC1 is regulated by ubiquitination of K446, suggesting that drugs targeting this ROR γ t ubiquitination pathway can inhibit ROR γ t activity by limiting co-activator SRC1 recruitment. Interestingly, a recent report shows that USP15 is up-regulated in psoriasis, supporting the possible role of USP15 in this Th17-mediated autoimmune disease (57). We expect that USP15 inhibitors will alleviate Th17-mediated autoimmunity including psoriasis via blocking the recruitment of SRC1. Although we identified USP15 as the deubiquitinase for K446, it remains unknown about the ubiquitin ligases responsible for K446 ubiquitination. Our future work is to search for the K446 E3 ligases which are also the potential drug target for controlling Th17 immunity. Therefore, in addition to revealing a new mechanism for ROR γ t-regulated Th17 differentiation, our results may facilitate the development of a new category of ROR γ t-based drugs that treat Th17-mediated autoimmunity by controlling ROR γ t ubiquitination-regulated recruitment of SRC1.

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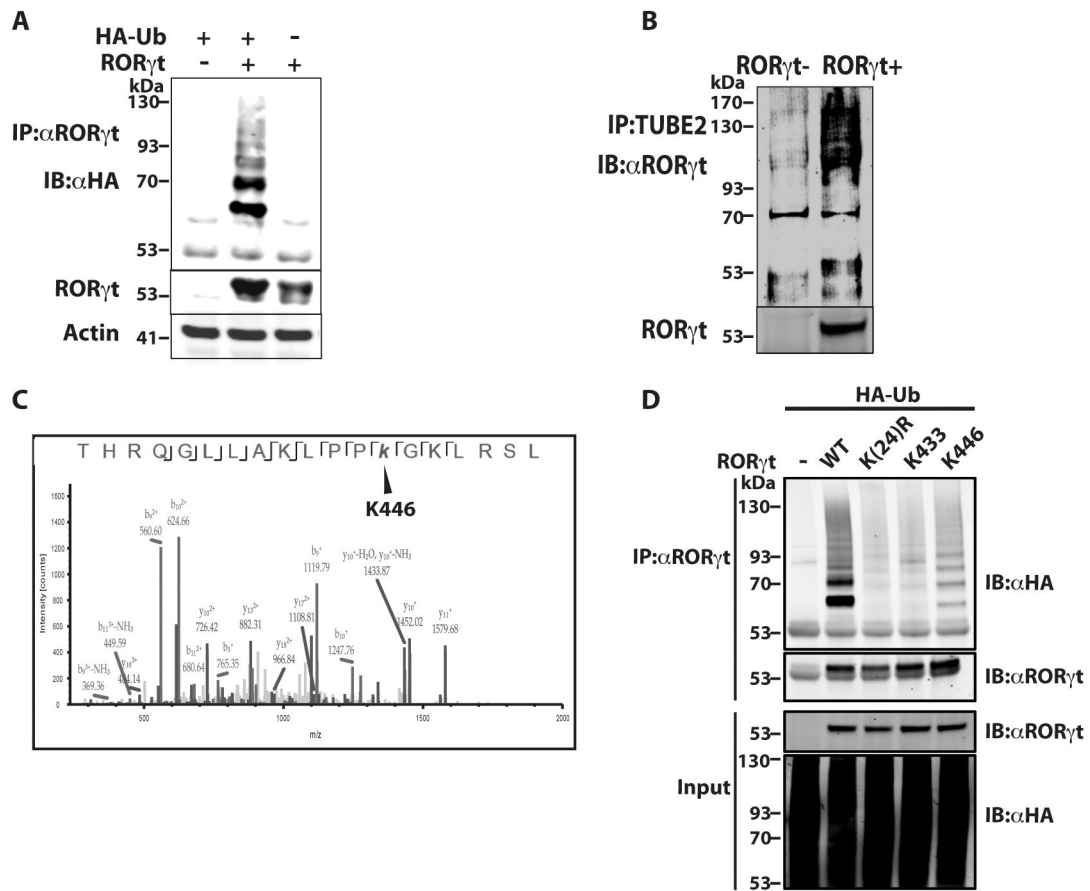
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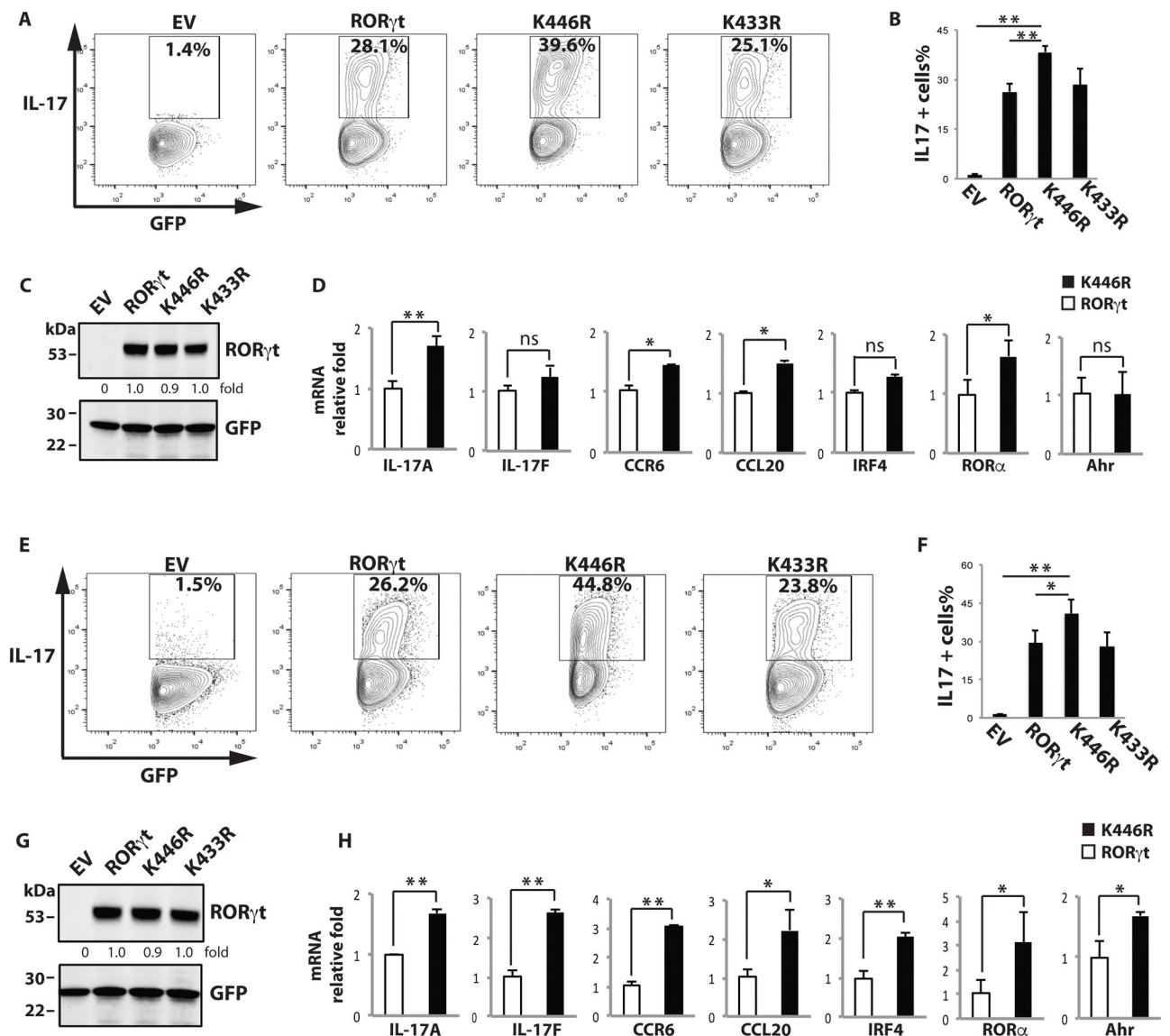
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**Figure 1.**

K446 of ROR γ t is a ubiquitination site. (A) Expression plasmids encoding ROR γ t and HA-Ub were transfected into 293T cells for 48 h. ROR γ t was then immunoprecipitated (IP) with anti-ROR γ t antibody (α ROR γ t) and resolved by SDS-PAGE. Ubiquitinated ROR γ t was detected by immunoblot (IB) with anti-HA antibody (α HA). Actin was used as a loading control. (B) Purified naïve CD4⁺ T cells were differentiated under Th17 priming conditions (TGF β and IL-6) for three days. Ubiquitinated proteins in the purified cell lysates were enriched by IP with TUBE beads and resolved on an SDS-PAGE gel. ROR γ t in the immunoprecipitated ubiquitinated proteins was then detected by IB with anti-ROR γ t antibody. (C) ROR γ t immunoprecipitated from differentiated Th17 cells was digested by trypsin and analyzed by MS. Shown are the MS/MS peptide spectra that identified K446 as a ubiquitination site. (D) Expression plasmids encoding ROR γ t or indicated ROR γ t mutants and HA-Ub were transfected into 293T cells for 48 h. ROR γ t was then IP with α ROR γ t and resolved by SDS-PAGE. Ubiquitinated ROR γ t was detected by IB with α HA. Data shown except (C) are the representatives of at least three independent experiments.

**Figure 2.**

K446 ubiquitination negatively regulates Th17 differentiation. (A–C) T cells obtained from *ROR γ t*^{-/-} mice were transduced with retrovirus expressing GFP only (empty virus, EV) or together with ROR γ t WT, K446R, or K433R, and differentiated under Th17-differentiating conditions. Three days later, intracellular IL-17 was detected by flow cytometry. Percentages of IL-17+ cells among GFP+ cells are indicated. **B** is the summary of three independent experiments. ROR γ t and GFP expression was detected by western blot analysis in **C**, and expression levels relative to wild type ROR γ t was calculated. (D) RNA was prepared from ROR γ t (open bars)- or K446R (black bars)-reconstituted *ROR γ t*^{-/-} T cells differentiated under Th17 conditions. qPCR was performed to quantitate expression of the indicated genes. * P < 0.05, ** P < 0.01. ns stands for not significant. (E–G) Differentiated Th17 cells described in (A) were rested in IL-2 for 48 h, placed in Th17 differentiation conditions for 3 more days, and analyzed as described in (A). **F** is the summary of three independent

experiments. ROR γ t and GFP expression was detected by westernblot analysis in **G**, and expression levels relative to wild type ROR γ t was calculated. **(H)** Critical Th17 gene expression in ROR γ t (open bars)- or K446R (black bars)-reconstituted *ROR γ t^{-/-}* T cells rested and redifferentiated as described in **(E)** was analyzed by qPCR. Unless specified, data shown are the representative of at least three independent experiments.

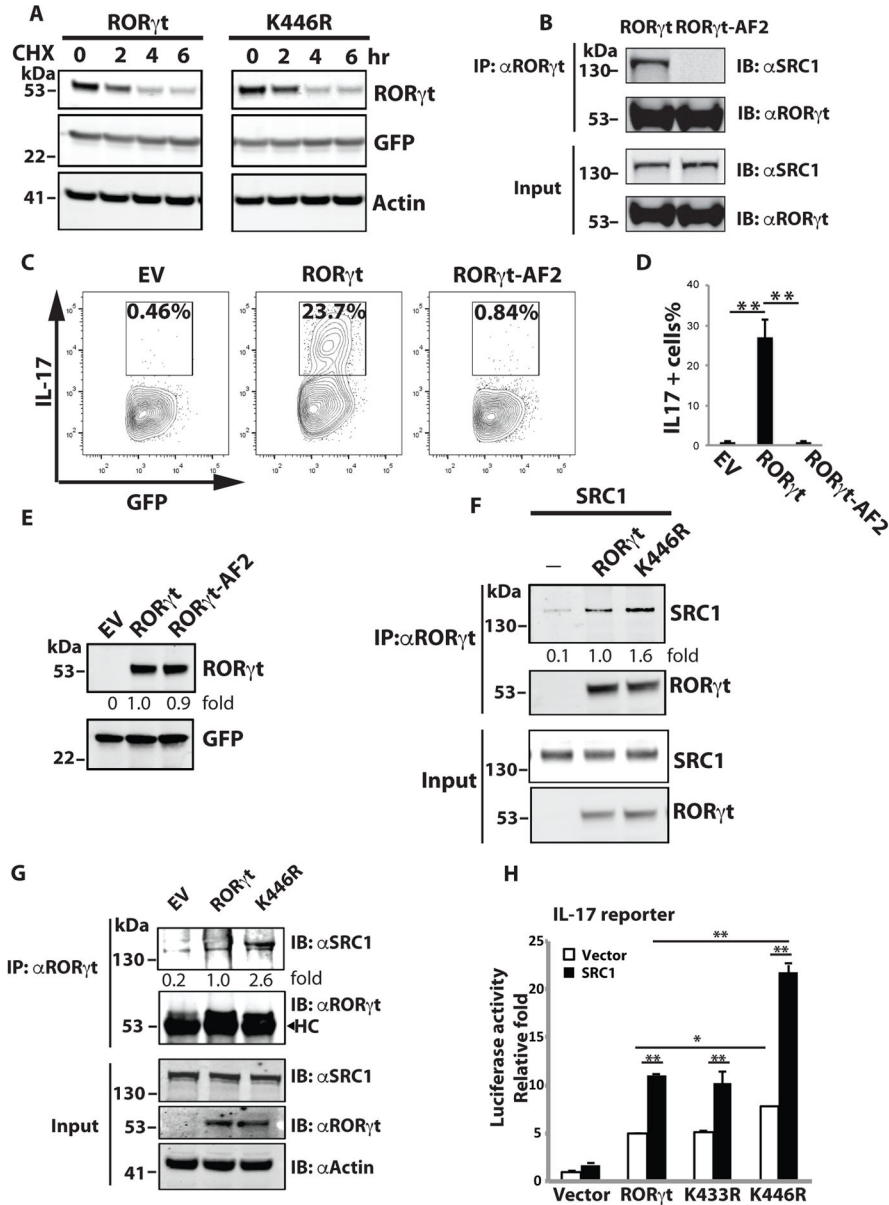


Figure 3. K446 ubiquitination impairs ROR γ t activity by reducing the recruitment of co-activator SRC1. (A) Expression plasmids for GFP together with ROR γ t or K446R were transfected into 293T cells for 48 h. Cells were then treated with protein synthesis inhibitor cycloheximide (CHX) for the indicated time (hr) and ROR γ t and GFP were detected by IB. (B) ROR γ t WT or AF2 mutant was expressed with SRC1 in 293T cells for 48 h. IP was performed to detect ROR γ t-SRC1 interactions. (C–E) ROR γ t^{-/-} T cells were transduced with retrovirus expressing GFP only (empty virus, EV) or together with ROR γ t WT or AF2 mutant, and differentiated under Th17 conditions. Three days later, intracellular IL-17 was detected by flow cytometry. Percentages of IL-17 positive cells among GFP+ cells are indicated. D is the summary of three independent experiments. ROR γ t and GFP expression

was detected by westernblot analysis in **E**. **(F)** ROR γ t WT or K446R was expressed with SRC1 in 293T cells for 48 h. IP was performed to detect ROR γ t-SRC1 interactions. Immunoprecipitated SRC1 levels relative to that immunoprecipitated by wild type ROR γ t was calculated. **(G)** ROR γ t WT or K446R was transduced into ROR γ t^{-/-} T cells differentiated under Th17 conditions for three days. IP was performed to detect ROR γ t-SRC1 interactions. Immunoprecipitated SRC1 levels relative to that immunoprecipitated by wild type ROR γ t was calculated. HC indicates heavy chain. **(H)** IL-17 promoter luciferase reporter was transfected into 293T cells with control or SRC1 plasmid and indicated ROR γ t mutants for 24 h. Luciferase assay was performed to quantitate relative reporter activity. Data represent mean \pm SE from three independent experiments with at least two replicas in each experiment. * <0.05 and ** <0.01 . Unless specified, data shown are the representative of at least three independent experiments.

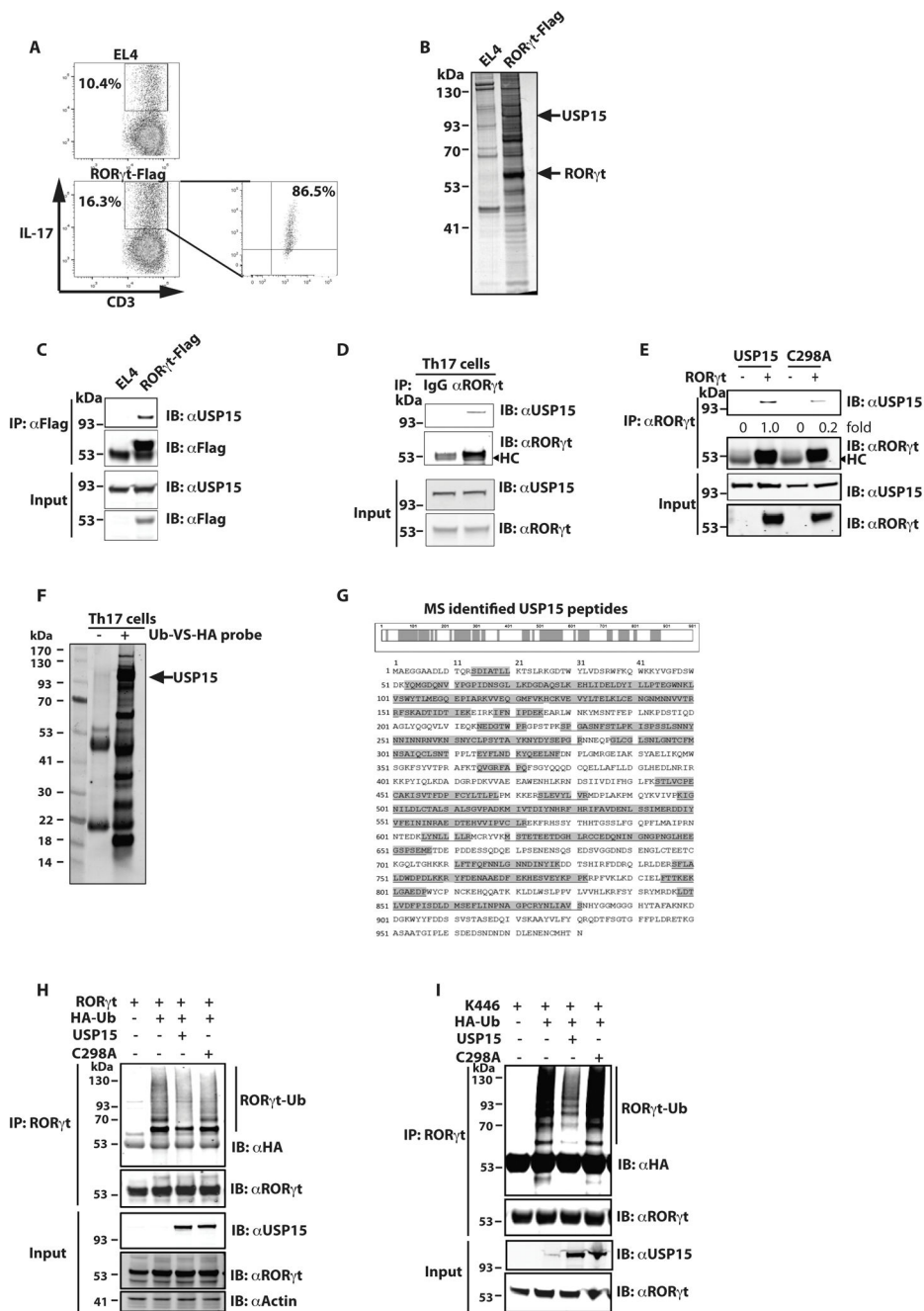


Figure 4.

USP15 interacts with ROR γ t and deubiquitinates K446. (A) Control or ROR γ t-flag expressing EL-4 cells were established and intracellular IL-17 was detected by flow cytometry. IL-17-producing cells in gated area were enriched by IL-17 catching beads and analyzed for intracellular IL-17 (right panel). (B) Anti-flag antibody was used to IP lysates from control or ROR γ t-flag-expressing EL-4 cells and IP complexes were resolved on a protein gel. Arrows indicate the bands containing USP15 and ROR γ t on the silver stained gel. (C) ROR γ t-SRC1 interaction was detected by IB of anti-flag antibody IP cell lysates of

control or ROR γ t-flag expressing EL-4 cells. **(D)** Naïve T cells were differentiated into Th17 cells for three days. Control IgG or α ROR γ t was used to perform IP to detect ROR γ t-USP15 interactions. HC indicates heavy chain. **(E)** WT USP15 or C298A was expressed with ROR γ t in 293T cells for 48 h. IP was performed to detect ROR γ t-USP15 interactions. Immunoprecipitated USP15 levels relative to that immunoprecipitated by wild type ROR γ t was calculated. HC indicates heavy chain. **(F)** Lysates of differentiated Th17 cells were incubated with or without Ub-HS-HA probe to covalently crosslink active deubiquitinases, which were then IP with α HA and resolved by SDS-PAGE. Arrow indicates the band containing USP15. **(G)** MS-identified USP15 peptides are highlighted by shadows. **(H)** ROR γ t was expressed alone or together with USP15 WT or inactive C298A mutant in the presence of HA-Ub in 293T cells for 48 h. ROR γ t was then IP and ubiquitinated ROR γ t (ROR γ t-Ub) was detected by IB with α HA. **(I)** K446 (all other Ks are mutated to Rs except K446) was expressed alone or together with USP15 WT or inactive C298A mutant in the presence of HA-Ub in 293T cells for 48 h. ROR γ t was then IP and ubiquitinated ROR γ t (ROR γ t-Ub) was detected by IB with α HA. Unless specified, data shown are the representative of at least three independent experiments.

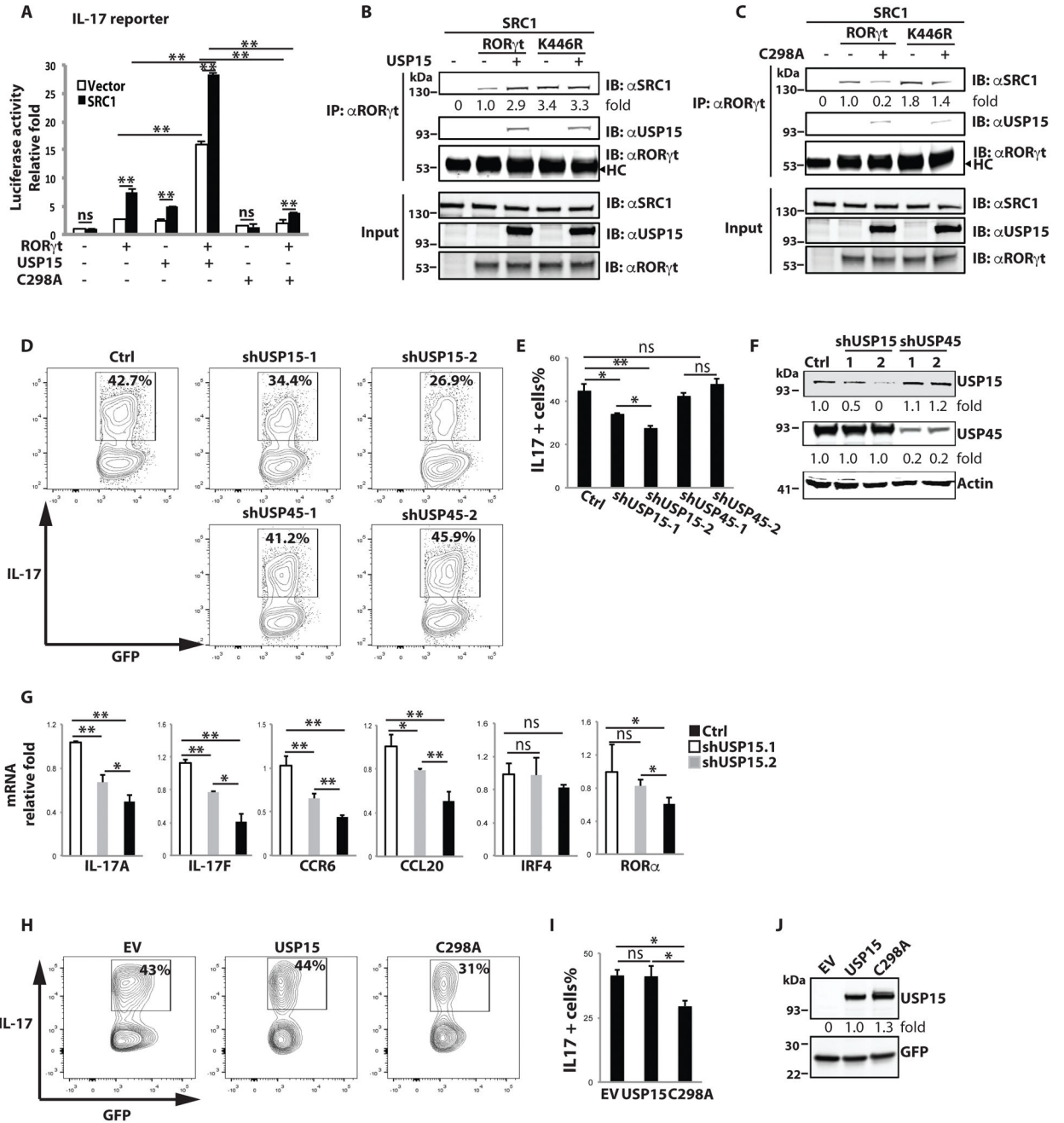


Figure 5.

USP15 regulates ROR γ t activity and Th17 differentiation via enhancing SRC recruitment. (A) IL-17 reporter was transfected into 293T cells together with vector (open bars) or SRC1 expression plasmid (black bars) and indicated plasmids for 24 h. Luciferase activity was then measured. Data represent mean \pm SE from three independent experiments with at least two replicas in each experiment. **<0.01. ns stands for not significant. (B) ROR γ t or K446R was expressed together with SRC1 in the presence or absence of USP15 in 293T cells for 48 h. IP with α ROR γ t was performed to detect ROR γ t-SRC1 interactions. Immunoprecipitated SRC1 relative to that immunoprecipitated by wild type ROR γ t was calculated. (C) ROR γ t or

K446R was expressed with SRC1 in the presence or absence of inactive USP15 C298A mutant in 293T cells for 48 h. IP with α ROR γ t was performed to detect ROR γ t-SRC1 interaction. Immunoprecipitated SRC1 relative to that immunoprecipitated by wild type ROR γ t was calculated. **(D–F)** Retrovirus expressing GFP with scrambled shRNA (Ctrl) or shRNAs specific for knockdown of USP15 or USP45 was transduced into CD4⁺ T cells and differentiated under Th17 conditions for three days. Intracellular IL-17 was detected by flow cytometer. **E** is the summary of three independent experiments. Knockdown of USP15 and USP45 were monitored by IB in **F**. **(G)** RNA was prepared from control (open bars), shUSP15-1 (grey bars)- or shUSP15-2 (black bars)-transduced T cells that differentiated under Th17 conditions. qPCR was performed to quantitate expression of the indicated genes. * P < 0.05, ** P<0.01. ns stands for not significant. **(H–J)** Retrovirus expressing GFP alone (EV) or together with USP15 WT or inactive C298A mutant were transduced into CD4⁺ T cells and differentiated under Th17 conditions for three days. Intracellular IL-17 was detected by flow cytometry. **(I)** is the summary of three independent experiments. USP15 and GFP expression was detected by westernblot analysis in **J**, and expression levels relative to wild type ROR γ t was calculated. Unless specified, data shown are the representative of at least three independent experiments.