Otud7b facilitates T cell activation and inflammatory responses by regulating Zap70 ubiquitination

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Signal transduction from the T cell receptor (TCR) is crucial for T cell–mediated immune responses and, when deregulated, also contributes to the development of autoimmunity. How TCR signaling is regulated is incompletely understood. In this study, we demonstrate a ubiquitin-dependent mechanism in which the deubiquitinase Otud7b has a crucial role in facilitating TCR signaling. Upon TCR ligation, Otud7b is rapidly recruited to the tyrosine kinase Zap70, a central mediator of TCR-proximal signaling. Otud7b deficiency attenuates the activation of Zap70 and its downstream pathways and impairs T cell activation and differentiation, rendering mice refractory to T cell–mediated autoimmune and inflammatory responses. Otud7b facilitated Zap70 activation by deubiquitinating Zap70, thus preventing the association of Zap70 with the negative-regulatory phosphatases Sts1 and Sts2. These findings establish Otud7b as a positive regulator of TCR-proximal signaling and T cell activation, highlighting the importance of deubiquitination in regulating Zap70 function.

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T cells are the central players of adaptive immune responses against infections and, when deregulated, are also responsible for autoimmune and inflammatory disorders (Ohashi, 2002). Upon stimulation by an antigen, naive T cells are activated to proliferate and subsequently differentiate into various effector T cells that participate in different aspects of immune functions (Smith-Garvin et al., 2009). In particular, activated $CD4^+$ T cells differentiate into several subsets of T helper cells, including Th1, Th2, Th17, and follicular T (Tfh) cells, as well as the immunosuppressive regulatory T (T reg) cells (Zhu et al., 2010). Naive T cell activation is initiated by the engagement of the TCR by a foreign antigen in the context of MHC molecules and also requires ligation of co-stimulatory molecules, such as CD28. The TCR–CD28 co-stimulation triggers cascades of signaling events, which regulate both the initial activation and the subsequent differentiation of T cells (Smith-Garvin et al., 2009).

TCR signaling initiates from activation of the protein tyrosine kinase Lck, which phosphorylates the TCR-signaling chain CD3ζ, leading to recruitment of the tyrosine kinase Zap70 to the TCR complex, in which Zap70 is phosphorylated and activated by Lck (Smith-Garvin et al., 2009). Activated Zap70 in turn phosphorylates several other signaling molecules, thereby transducing the TCR signal to various downstream signaling events, including activation of IκB kinase (IKK), MAP kinases, and several families of transcription factors. Consequently, these signaling events induce the production of cytokines, such as IL-2 and IFN-γ, and expansion of the T cells. The strength of the TCR signal has an important impact on the nature and magnitude of an immune response and is, therefore, subject to tight regulation by both positive and negative mechanisms.

Ubiquitination is an important mechanism that regulates T cell activation and immune responses (Liu et al., 2005). Several E3 ubiquitin ligases, including c-Cbl, Cbl-b, GRAIL, and Itch, have been shown to negatively regulate TCR–CD28 signaling and prevent deregulated T cell activation and development of autoimmune diseases (Huang and Gu, 2008; Park et al., 2014). A major action of these E3s is to mediate ubiquitin-dependent degradation of TCR-signaling components, such as the TCR signaling chain TCRζ, protein kinase C θ, phospholipase C γ1, and PI3 kinase (Heiss-

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Abbreviations used: DUB, deubiquitinase; EAE, experimental autoimmune encephalomyelitis; HA, hemagglutinin; IB, immunoblot; ICS, intracellular cytokine staining; IKK, IκB kinase; IP, immunoprecipitation; LLO, listeriolysin O; MOG, myelin oligodendrocyte glycoprotein; qRT-PCR, quantitative RT-PCR; Traf, TNF receptor–associated factor; UBA, ubiquitin association.

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meyer et al., 2004; Huang and Gu, 2008; Park et al., 2014). However, accumulating evidence suggests that ubiquitination may also regulate the function of some TCR-signaling molecules without causing their degradation (Jeon et al., 2004; Huang et al., 2010). Precisely how nondegradative ubiquitination regulates TCR-proximal signaling events is poorly defined. Nevertheless, it has been proposed that the protein tyrosine phosphatase Sts1 (also called TULA-2 or Ubash3b) and its homologue, Sts2 (also called TULA or Ubash3a), may target substrates that are dually modified by ubiquitination and tyrosine phosphorylation (Carpino et al., 2009). Sts1 and Sts2 contain a ubiquitin-association (UBA) domain, an SH3 domain, and a phosphatase domain (Carpino et al., 2004), and one well-characterized substrate of these phosphatases is Zap70 (Carpino et al., 2004). However, it is currently unclear how Sts1/2 is recruited to Zap70 and whether ubiquitination plays a role.

Although ubiquitination is known to be important for regulating T cell activation and several E3 ubiquitin ligases have been characterized, little is known about the role of deubiquitinases (DUBs) in the regulation of TCR-proximal signaling. DUBs are proteases that cleave ubiquitin chains and counteract the action of E3 ligases (Sun, 2008). The mammalian genome encodes ~100 DUBs, suggesting a significant level of functional specificity. In addition to their differences in ubiquitin chain-specificity, DUBs contain distinct protein interaction domains and target specific substrates (Reyes-Turcu et al., 2009). We have previously demonstrated that a UBA domain-containing DUB, Otud7b, specifically targets a member of the TNF receptor–associated factor (Traf) family, Traf3 (Hu et al., 2013). Otud7b inhibits ubiquitin-dependent Traf3 degradation in B cells stimulated through TNF receptor family members, such as BAFF receptor and CD40, and, thereby, negatively regulates noncanonical NF-κB signaling and B cell activation (Hu et al., 2013). Because Traf3 has opposing roles in the regulation of B and T cell activation (Xie et al., 2007, 2011; Gardam et al., 2008), it raises the question of whether Otud7b also functions in T cells.

In this study, we obtained biochemical and genetic evidence that Otud7b is a crucial and positive regulator of TCR-proximal signaling. Otud7b deficiency attenuated TCR–CD28-stimulated activation of Zap70 and downstream signaling factors and impaired T cell activation and Th1 cell differentiation. Consistently, Otud7b-deficient mice were refractory to the induction of T cell–mediated autoimmunity and inflammation. Regarding the mechanism underlying Otud7b function, we did not detect Traf3 degradation in either WT or Otud7b-deficient T cells. Instead, we identified Zap70 as a specific target of Otud7b in the TCR pathway. In response to TCR–CD28 ligation, Otud7b rapidly bound to Zap70 and inhibited Zap70 ubiquitination. We further demonstrated that by deubiquitinating Zap70, Otud7b prevented the association of Zap70 with the phosphatase Sts1/2, thereby facilitating Zap70 phosphorylation and TCR signaling. Our data reveal a novel mechanism that regulates TCR signaling and establish Otud7b as a DUB that regulates T cell activation and T cell–mediated immune and autoimmune responses.

RESULTS

Otud7b regulates T cell homeostasis

The size of the peripheral T cell pool is maintained stably under normal conditions, a mechanism termed homeostasis (Takada and Jameson, 2009; Sprent and Surh, 2011). Although the majority of peripheral T cells are in a naive state, characterized by $CD44^{lo}CD62L^{hi}$ markers, a proportion of memory-like T cells (with $CD44^{hi}CD62L^{lo}$ markers) exists and increases to high levels at older ages, which is thought to result from homeostatic proliferation induced via TCR stimulation by self-peptide/MHC ligands (Takada and Jameson, 2009; Sprent and Surh, 2011). We have previously shown that Otud7b deficiency has no effect on the development of T or B cells (Hu et al., 2013), but it has remained unknown whether Otud7b plays a role in regulating the homeostasis of peripheral T cells. We addressed this question by analyzing the frequency of naive and memory-like T cells in the peripheral lymphoid organs of both young and older mice. Young adult mice (8 wk of age) with homozygous germline deletion of *Otud7b* ($Otud7b^{-/-}$) and WT control ($Otud7b^{+/+}$) mice had comparable frequency of naive and memory-like T cells (Fig. 1, A and B). However, at an older age (12 mo old), the $Otud7b^{-/-}$ mice had significantly reduced frequency and absolute numbers of memory-like T cells and concomitantly increased frequency and numbers of naive T cells in both $CD4^+$ and $CD8^+$ T cell populations (Fig. 1, C and D). Among the $CD44^{hi}$ memory T cell population in the spleen, IFN-γ–producing Th1 cell subset was profoundly reduced in the $Otud7b^{-/-}$ mice, whereas the Th17 cell subset was not appreciably affected (Fig. 1 E). The Otud7b deficiency also did not alter the frequency of T reg cells (Fig. 1 F). Similar results were obtained with lamina propria T cells, showing reduced frequency of Th1, but not Th17 or T reg, cells (Fig. 1, G and H). These results suggest that Otud7b plays an important role in regulating the homeostasis of T cells, particularly the production of the Th1 subset of effector/memory-like $CD4^+$ T cells.

Otud7b is crucial for antigen-specific and lymphopenic T cell responses

As stated above, homeostatic generation of memory-like T cells involves TCR signaling stimulated by self-peptide-MHC ligands (Jameson, 2005; Takada and Jameson, 2009; Sprent and Surh, 2011). The reduced frequency of memory-like T cells in older $Otud7b^{-/-}$ mice prompted us to examine the role of Otud7b in regulating T cell activation. We used an in vivo T cell response model involving challenging the mice with an intracellular bacterial pathogen, *Listeria monocytogenesis*, which is known to induce strong T cell responses characterized by the induction of IFN-γ–producing $CD4^+$ Th1 cells and $CD8^+$ effector T cells (Wakil et al.,

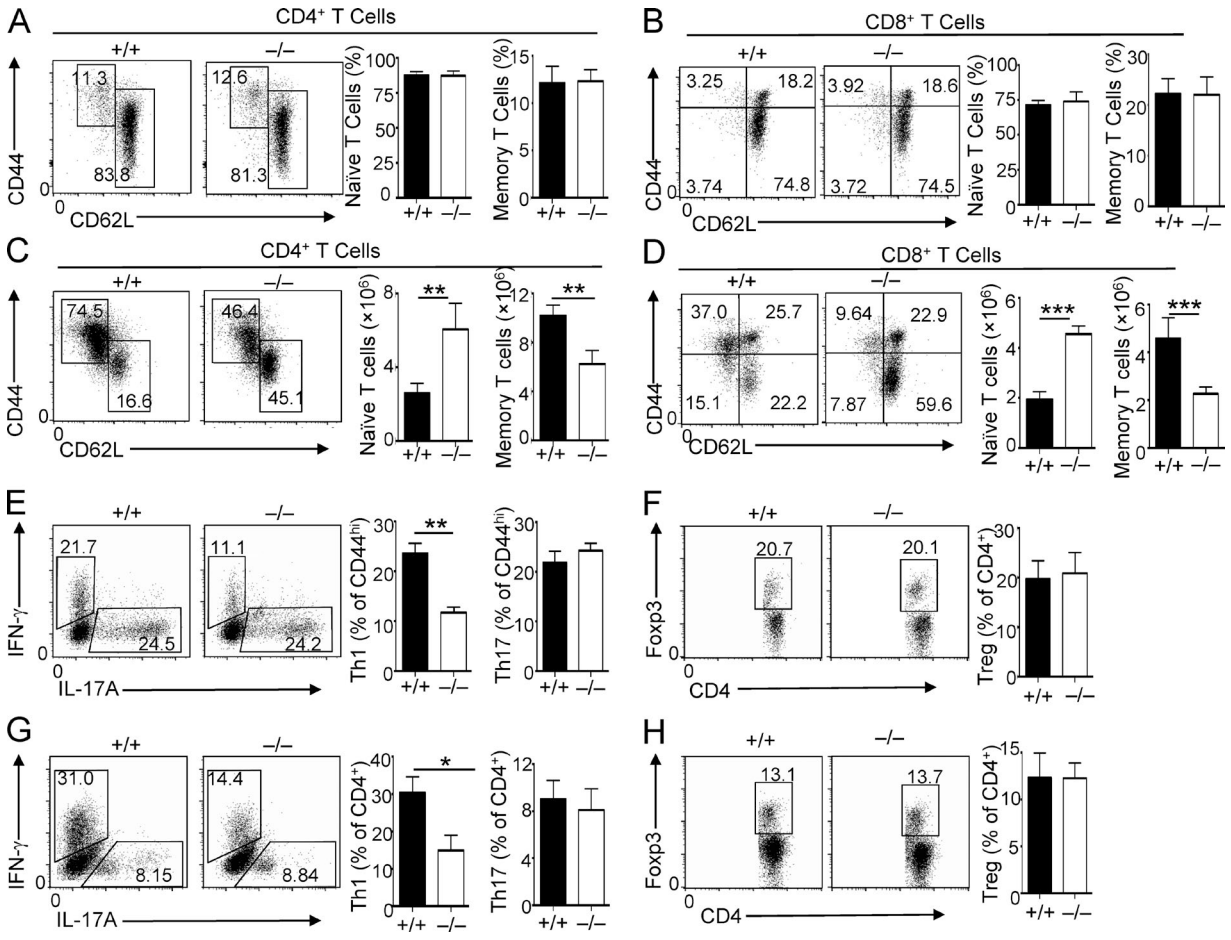

Figure 1. **Otud7b deficiency perturbs T cell homeostasis in older mice.** (A–D) Flow cytometry analyses of the percentage of naive ($CD44^{lo}CD62L^{hi}$) and memory ($CD44^{hi}CD62L^{lo}$) $CD4^+$ T cells (A and C) or the percentage of naive ($CD44^{lo}$) and memory ($CD44^{hi}$) $CD8^+$ T cells (B and D) in the spleen of young (A and B; 6–8-wk-old) or older (C and D; 12-mo-old) *Otud7b*$^{+/+}$ (+/+) and *Otud7b*$^{-/-}$ (–/–) mice. Data are presented as representative plots (left) and summary graphs of the mean ± SD values of three independent experiments ($n = 4$ for each experiment; right). (E–H) ICS and flow cytometry analyses of Th1 (IFN-γ^+) and Th17 (IL-17A$^+$) effector cells (E and G) or T reg cells (Foxp3$^+$; F and H) in the spleen (E and F) or lamina propria of small intestine (F and H) of *Otud7b*$^{+/+}$ and *Otud7b*$^{-/-}$ mice (12 mo old). Data are percentage of $CD4^+CD44^+$ cells (E and F) or total $CD4^+$ T cells (G and H) and presented as representative plots (left) and summary graphs of three independent experiments ($n = 4$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

1998; Lara-Tejero and Pamer, 2004). We chose to use young adult mice, as they had no obvious abnormalities in T cell homeostasis (Fig. 1, A and B). We infected *Otud7b*$^{-/-}$ and *Otud7b*$^{+/+}$ mice with a modified *L. Monocytogenes* strain encoding chicken OVA (LM-OVA; Foulds et al., 2002). 7 d after infection, we isolated splenocytes and restimulated the antigen-specific effector T cells with an MHC class II–restricted listeriolysin O peptide ($LLO_{190-201}$) and a MHC class I–restricted OVA peptide ($OVA_{257-264}$). As expected, LM-OVA infection of *Otud7b*$^{+/+}$ mice led to the generation of abundant $CD4^+$ and $CD8^+$ IFN-γ–producing effector T cells, as detected by restimulation with $LLO_{190-201}$ and $OVA_{257-264}$, respectively (Fig. 2, A and B). Importantly, the Otud7b deficiency greatly inhibited the generation of these effector T cells. Consistently, the *Otud7b*$^{-/-}$ mice had a significantly higher bacterial load in the liver (Fig. 2 C). These results demonstrated an important role for Otud7b in regulating T cell responses to bacterial infections.

To further examine the role of Otud7b in the regulation of in vivo T cell responses, we used a T cell transfer model. Transfer of naive $CD4^+$ T cells into the lymphocyte-deficient *Rag1*$^{-/-}$ mice is a frequently used approach to study inflammatory T cell responses under lymphopenic conditions. The transferred naive $CD45RB^{hi}$ $CD4^+$ T cells differentiate into inflammatory Th1 and Th17 cells that induce colon inflammation associated with bodyweight loss (Morrissey et al., 1993). As expected, transfer of WT $CD4^+CD25^-CD45RB^{hi}$ naive T cells into *Rag1*$^{-/-}$ mice caused severe colitis, characterized by gradual bodyweight loss (Fig. 2 D) and hyperplasia of the colonic mucosa (Fig. 2 E). In contrast, the Otud7b-deficient $CD4^+CD25^-CD45RB^{hi}$ naive T cells only induced moderate bodyweight loss and histological changes in the

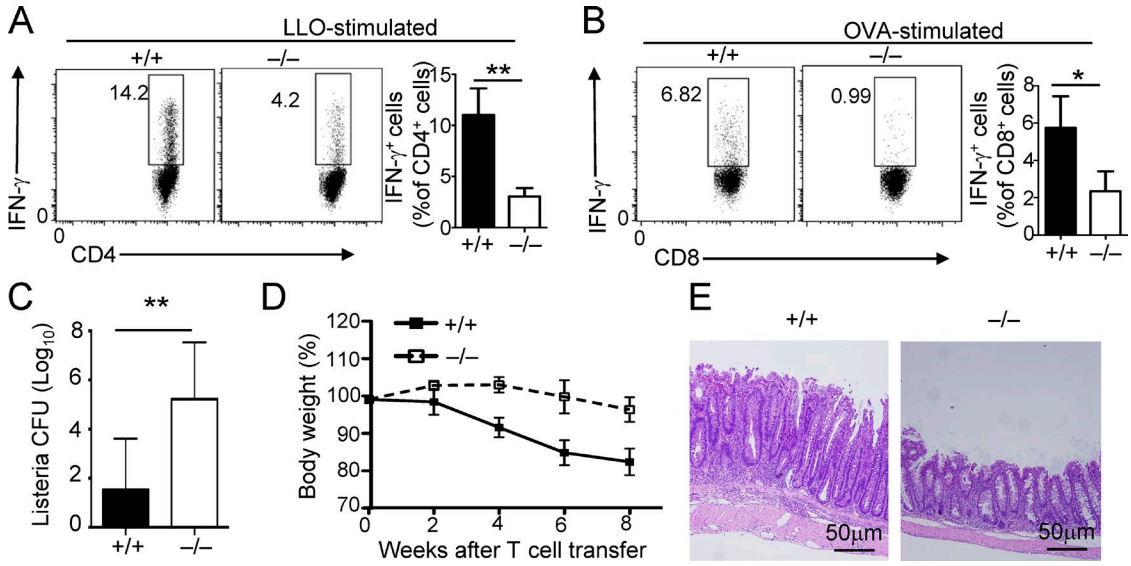

Figure 2. **Otud7b is required for T cell responses to bacterial infection and lymphopenic conditions.** (A and B) ICS and flow cytometry analyses of IFN-γ–producing CD4$^+$ and CD8$^+$ T cells in the spleen of WT and *Otud7b*$^{-/-}$ mice infected with LM-OVA for 7 d. Splenocytes were restimulated for 5 h with LLO190-201 (A) or OVA257-264 (B) peptide in the presence of monesin before the analyses, and data are presented as a representative plot (left) or summary graph (right). (C) *L. monocytogenes* titer in the liver, expressed as CFUs. (D and E) Bodyweight loss (D; percent of starting bodyweight) and colon histology (E; H&E staining) of *Rag1*$^{-/-}$ mice adoptively transferred with WT or *Otud7b*$^{-/-}$ CD4$^+$CD45RB^{hi} T cells for the indicated time periods (D) or 8 wk (E). Data in all panels are representative of three independent experiments (n = 5). *, P < 0.05; **, P < 0.01.

colon (Fig. 2, D and E). Collectively, these results suggest that Otud7b is required for T cell responses under both pathogen infection and lymphopenic conditions.

Otud7b deficiency ameliorates pathogenesis of a T cell–dependent autoimmunity

T cell responses to self-antigens contribute to the development of many autoimmune and inflammatory diseases. To determine the role of Otud7b in regulating autoimmune inflammatory T cell responses, we performed experimental autoimmune encephalomyelitis (EAE), a T cell–mediated autoimmunity that mimics the human neuroinflammatory disease multiple sclerosis (Robinson et al., 2014). The pathogenesis of EAE involves priming of autoimmune T cells in the peripheral lymphoid organs and infiltration of the generated Th1 and Th17 subsets of inflammatory T cells into the CNS (El-behi et al., 2010; Zepp et al., 2011). As expected, immunization of *Otud7b*$^{+/+}$ mice with a peptide derived from a CNS protein, myelin oligodendrocyte glycoprotein (MOG), induced severe EAE disease symptoms. Compared with the *Otud7b*$^{+/+}$ mice, the *Otud7b*$^{-/-}$ mice had delayed onset and reduced clinical scores of EAE, suggesting a role for Otud7b in facilitating EAE induction (Fig. 3 A). Consistently, the *Otud7b*$^{-/-}$ mice had a substantially reduced frequency and absolute number of CNS-infiltrating CD4$^+$ and CD8$^+$ T cells and CD11b$^+$ monocytes during the early effector phase of EAE (day 14 after immunization; Fig. 3, B and C). These mutant animals also had a reduced percentage of IFN-γ–producing Th1 cells, although not IL-17A–producing Th17 cells, within the CNS-infiltrating CD4$^+$ T cell population (Fig. 3, D and E). Furthermore, the absolute number of both Th1 and Th17 cells infiltrating the CNS of the *Otud7b*$^{-/-}$ mice was drastically reduced (Fig. 3 F), which was apparently due to the reduction in CNS-infiltrating total CD4$^+$ T cells (Fig. 3 C).

To determine whether Otud7b regulated the production of inflammatory effector T cells, we analyzed the frequency of effector T cells in the draining LN and spleen of the MOG-immunized mice. Compared with *Otud7b*$^{+/+}$ mice, the *Otud7b*$^{-/-}$ mice had reduced frequencies of Th1, but not Th17, cells in both of the spleen and draining LN (Fig. 3, D and E). This result was consistent with the reduced frequency of Th1 cells in the CNS of the *Otud7b*$^{-/-}$ mice (Fig. 3, D and E). Parallel flow cytometry analyses revealed similar frequencies of cells in both the peripheral lymphoid organs and the CNS of the MOG-immunized *Otud7b*$^{-/-}$ and *Otud7b*$^{+/+}$ mice (unpublished data).

To assess the role of Otud7b in regulating the recall response of the antigen-specific T cells, we stimulated T cells isolated from the spleen and draining LNs of *Otud7b*$^{-/-}$ and *Otud7b*$^{+/+}$ mice at their early effector phase of EAE induction (day 10). Upon restimulation with MOG peptide, the T cells derived from *Otud7b*$^{+/+}$ mice underwent vigorous proliferation, whereas the T cells derived from *Otud7b*$^{-/-}$ mice had profound defect in such a recall response (Fig. 3 G). Consistently, the Otud7b-deficient T cells also produced less cytokines, including the T cell growth factor IL-2 and signature cytokines for Th1 (IFN-γ) and Th17 (IL-17A) cells (Fig. 3 H). The defect in IFN-γ induction might be partially

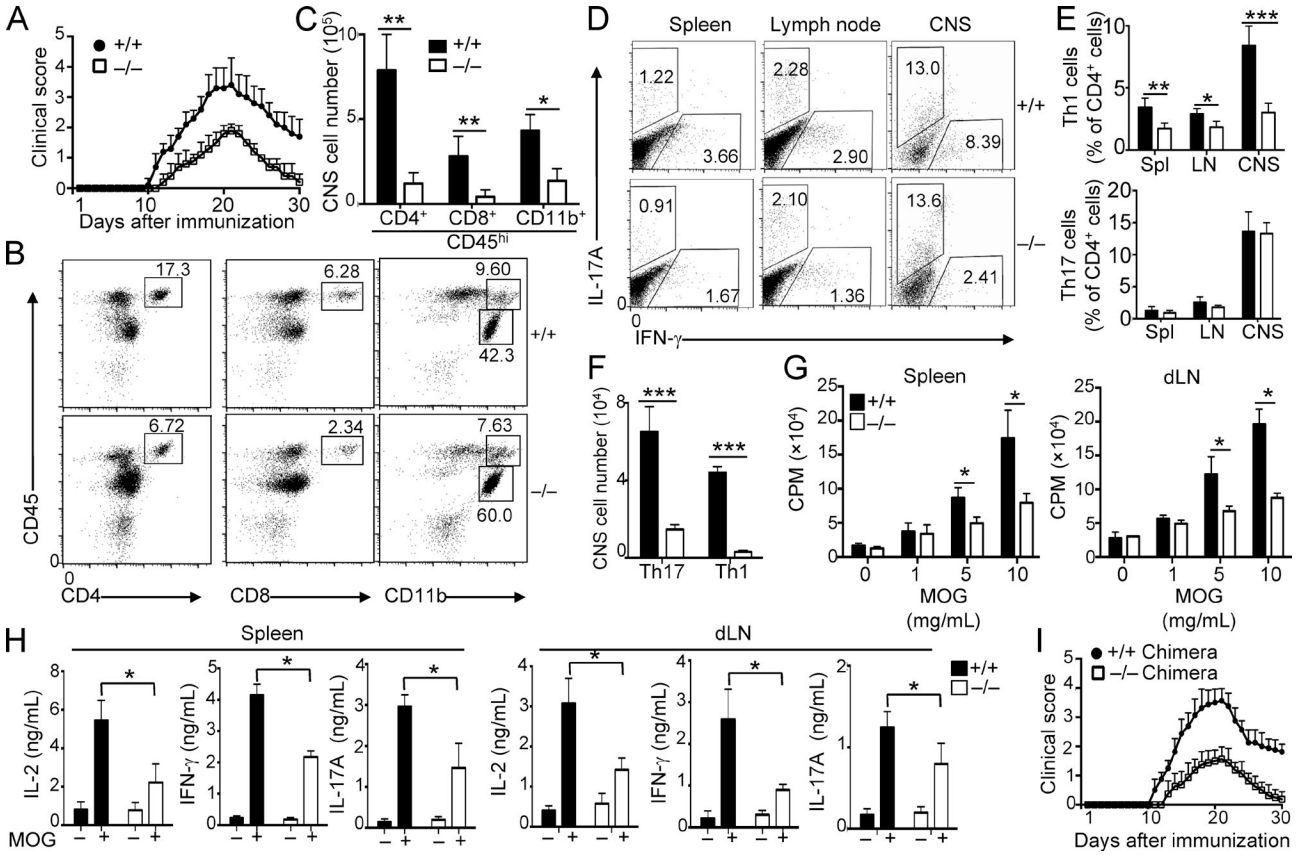

Figure 3. **Otud7b deficiency attenuates T cell activation and ameliorates EAE induction.** (A) Mean clinical scores of WT and *Otud7b*$^{-/-}$ mice after EAE induction by MOG_{35-55} immunization. The data represent two independent experiments ($n = 8$ for each experiment). (B and C) Flow cytometry analysis of infiltrating CD4$^+$ and CD8$^+$ T cells, infiltrating monocytes (CD11b$^+$CD45^{hi}), and resident microglia (CD11b$^+$CD45^{lo}) in the CNS of day 14 EAE-induced *Otud7b*$^{+/+}$ and *Otud7b*$^{-/-}$ mice, showing a representative plot (B) and a summary graph (C). (D–F) ICS and flow cytometry analysis of IFN-γ$^+$ Th1 and IL-17A$^+$ Th17 cells in the spleen, draining LN, and CNS of EAE-induced (day 14) WT and Otud7b$^{-/-}$ mice. Data are presented as a representative plot (D) and summary graphs of the percentage (E) or absolute numbers (F) of Th1 and Th17 cells. (G and H) Recall responses of antigen-specific T cells in the splenocytes and draining LN (dLN) cells of EAE-induced (day 14) *Otud7b*$^{+/+}$ and *Otud7b*$^{-/-}$ mice. Splenocytes and draining LN cells were stimulated with the indicated concentration (G) or 10 µg/ml (H) of MOG peptide for 2 d and subjected to cell proliferation assay (G; based on [^{3}H]-thymidine incorporation) and ELISA analyses (H). Data in B–H are representative of two independent experiments ($n = 3$ for each experiment). (I) Mean clinical scores of WT and Otud7b$^{-/-}$ chimeric mice (*TCRβ/δ*$^{-/-}$ mice adoptively transferred with WT or *Otud7b*$^{-/-}$ bone marrows mixed with *TCRβ/δ*$^{-/-}$ bone marrows) induced for EAE by MOG_{35-55} immunization ($n = 6$). Data are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

caused by the reduced frequency of Th1 cells in the lymphoid organs of *Otud7b*$^{-/-}$ mice (Fig. 3 E). However, because the *Otud7b*$^{-/-}$ and *Otud7b*$^{+/+}$ mice had similar frequencies of Th17 cells, the defect of the Otud7b-deficient T cells in IL-17A induction suggested that these mutant T cells had a defect in recall responses. This possibility was also supported by the reduced induction of IL-2 in the Otud7b-deficient T cells.

We next examined whether the EAE-regulatory function of Otud7b was T cell–intrinsic by performing mixed bone marrow adoptive transfer experiments, in which we adoptively transferred irradiated T cell deficient *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice with a mixture of *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ bone marrow and *Otud7b*$^{-/-}$ (or *Otud7b*$^{+/+}$) bone marrow (in a 3:1 ratio). Because the *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ bone marrow produces all immune cells except T cells, the only cell type lacking Otud7b in the *Otud7b*$^{-/-}$ mixed bone marrow chimeric mice should be T cells. The recipients of *Otud7b*$^{-/-}$ plus *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ bone marrows (called *Otud7b*$^{-/-}$ chimera) and the recipients of *Otud7b*$^{+/+}$ plus *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ bone marrows (called *Otud7b*$^{+/+}$ chimera) had comparable thymocyte and peripheral T cell populations, indicative of similar efficiency of thymocyte development (not depicted). However, the *Otud7b*$^{-/-}$ chimeric mice were substantially more resistant to EAE induction than the *Otud7b*$^{+/+}$ chimeric mice, thus further emphasizing a T cell–intrinsic role for Otud7b in regulating EAE pathogenesis (Fig. 3 I).

Otud7b mediates T cell activation and differentiation in vitro

To more directly examine the function of Otud7b in regulating T cell activation, we used an in vitro approach by stimulating naive CD4$^+$ T cells with agonistic antibodies for

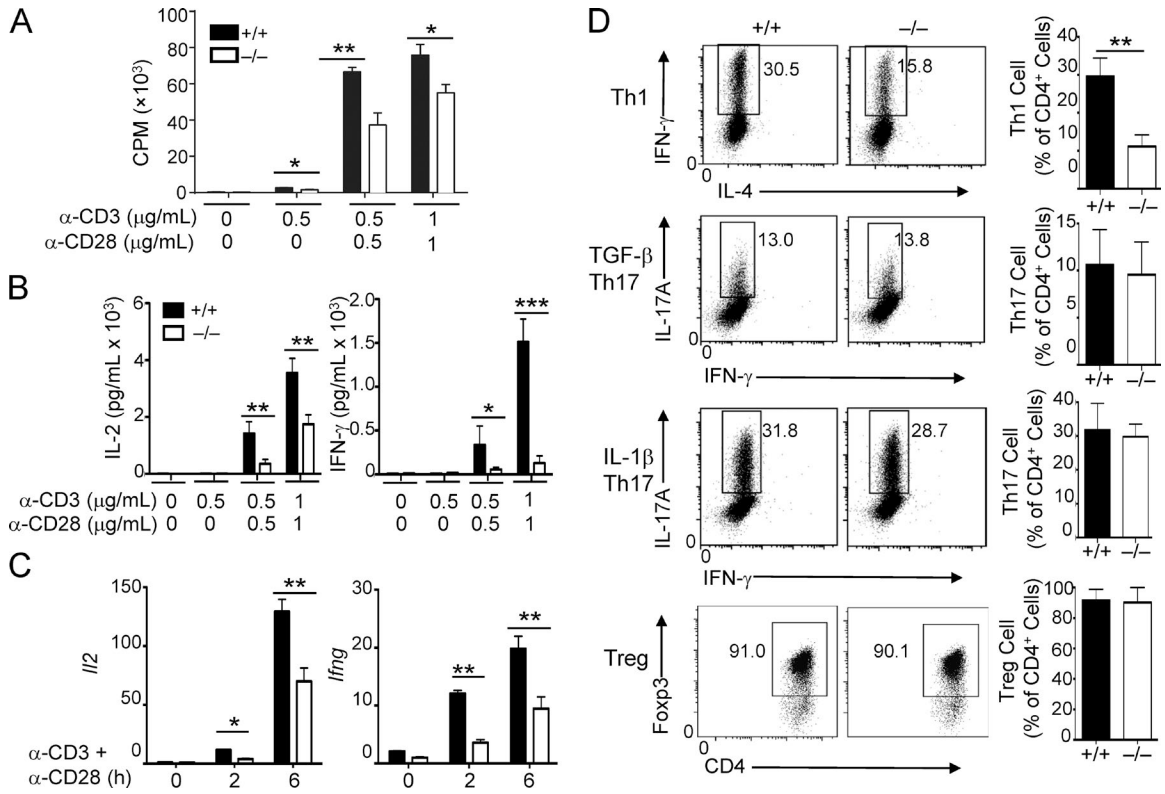

Figure 4. **Otud7b regulates T cell activation and Th1 cell differentiation in vitro.** (A and B) Cell proliferation based on [^{3}H]-thymidine incorporation (A) and ELISA analyses of the indicated cytokines in the supernatants (B) of WT or *Otud7b*$^{-/-}$ naive CD4$^+$ splenic T cells stimulated for 40 h with the indicated doses of agonistic anti-CD3 and anti-CD28 antibodies. (C) qRT-PCR analyses of WT or *Otud7b*$^{-/-}$ naive T cells stimulated with anti-CD3 and anti-CD28 antibodies for the indicated time periods. (D) ICS and flow cytometry analyses of Th1 cells, Th17 cells differentiated under TGF-β– and IL-1β–stimulating conditions, and T reg cells generated by in vitro differentiation of naive CD4$^+$ T cells, presented as representative plots (left) and summary graphs of mean ± SD values of multiple mice (right; *n* = 4). Data in all panels are representative of three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

TCR (anti-CD3) and CD28 (anti-CD28) and measuring T cell responses based on proliferation and cytokine production. When stimulated with anti-CD3 or anti-CD3 plus anti-CD28, Otud7b-deficient T cells were attenuated in proliferation, as revealed by a significant lower level of thymidine incorporation than the WT T cells (Fig. 4 A). Parallel ELISA revealed a profound reduction of the *Otud7b*$^{-/-}$ T cells in producing two major cytokines, IL-2 and IFN-γ (Fig. 4 B). The defect of the *Otud7b*$^{-/-}$ T cells in cytokine production was also detected at the mRNA level, indicating impaired induction of the *Il2* and *Ifng* genes (Fig. 4 C). These results confirmed the role of Otud7b in regulating T cell activation.

After their initial activation, CD4$^+$ T cells differentiate into different subsets of helper T cells and T reg cells, which can be detected based on their cytokine production profile and expression of the key signature transcription factor. The in vivo differentiation of CD4$^+$ T cells is regulated by both cytokines present in the microenvironment and T cell–intrinsic factors. Given the crucial involvement of Otud7b in regulating T cell activation in vitro and inflammatory T cell responses in vivo, we surmised that Otud7b might have a T cell–intrinsic role in regulating T cell differentiation. We used an in vitro T cell differentiation system, in which naive CD4$^+$ T cells were stimulated through the TCR and CD28 under different polarizing conditions. We found that the Otud7b-deficient T cells displayed a defect in Th1 cell generation (Fig. 4 D), a result that was in line with the finding that *Otud7b*$^{-/-}$ mice had reduced production of Th1 cells in response to bacterial infection and MOG-mediated EAE induction (Fig. 2, A and B and Fig. 3 E). In contrast, the Otud7b deficiency did not affect CD4$^+$ T cell differentiation to Th17 cells under either TGF-β– or IL-1β–polarizing conditions (Fig. 4 D). Similarly, the *Otud7b*$^{-/-}$ T cells were also competent in the in vitro generation of inducible T reg cells (Fig. 4 D). These results suggest that Otud7b is an important factor that regulates both T cell activation and CD4$^+$ T cell differentiation, particularly the generation of inflammatory Th1 cells.

Otud7b facilitates TCR signaling

The cell-intrinsic functions of Otud7b in mediating T cell activation and differentiation suggested a role for Otud7b in regulating TCR signaling. We tested this possibility using T cells isolated from the spleen and LNs of young adult (6–8-wk-old) mice that had no obvious abnormalities in T cell homeosta-

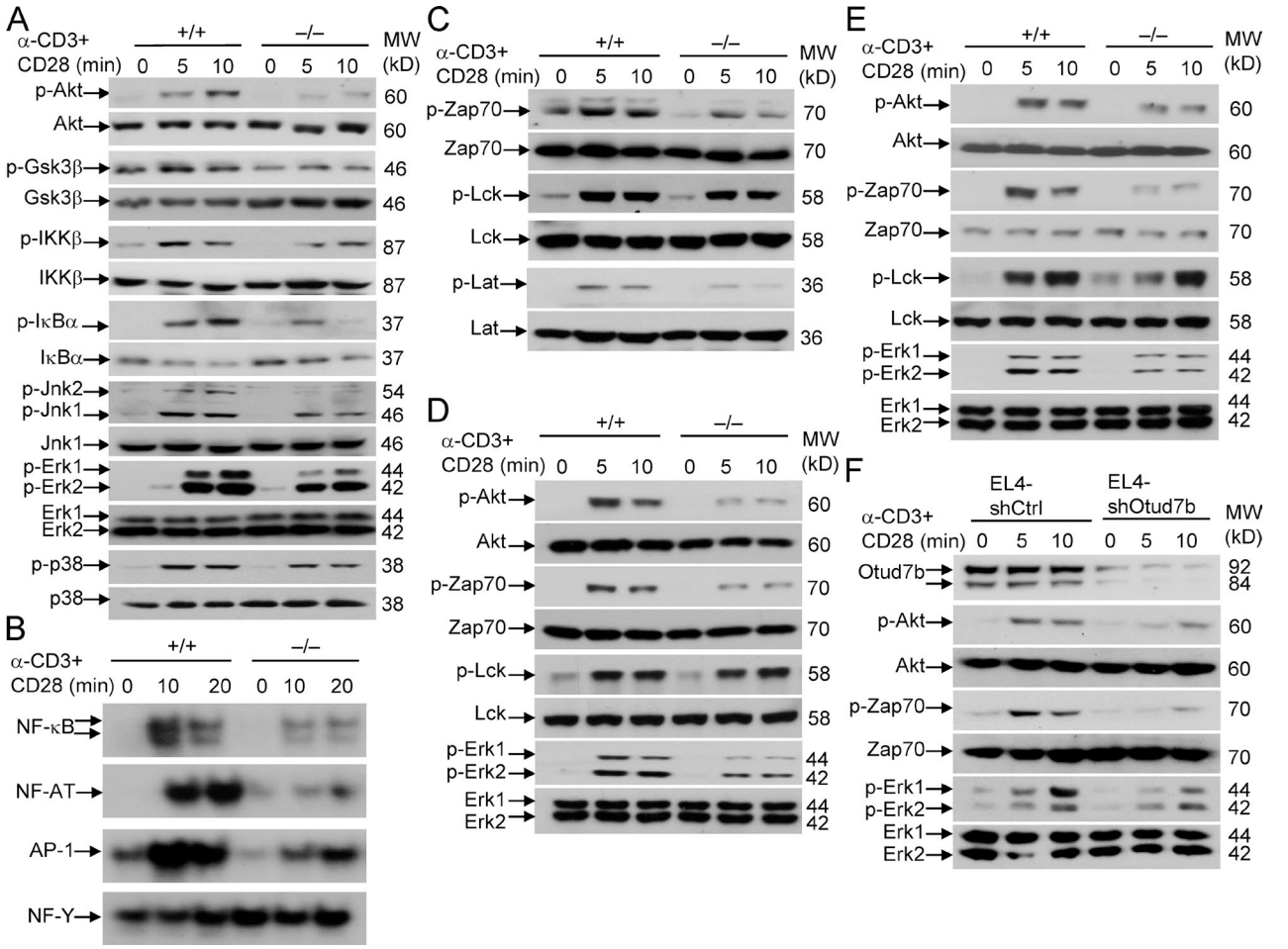

Figure 5. ***Otud7b*$^{-/-}$ T cells have impaired TCR signaling.** (A–E) IB analyses of the indicated phosphorylated (p-) and total proteins (A and C–E) and EMSA analysis of the indicated transcription factors in the nuclear extracts (B) of total T cells (A–C) or naive CD4$^+$ (D) and naive CD8$^+$ (E) T cells acutely stimulated with anti-CD3 plus anti-CD28 using an antibody cross-linking method. (F) IB analysis using whole-cell extracts of EL4 cells transduced with a nonsilencing shRNA control (EL4-shCtrl) or an Otud7b-specific shRNA (EL4-shOtud7b), acutely stimulated with anti-CD3 plus anti-CD28 using an antibody-cross-linking method. Data in all panels are representative of three independent experiments.

sis. Cross-linking of the TCR and CD28 stimulates proximal signaling events, including tyrosine phosphorylation of Lck and Zap70, leading to activation of multiple downstream kinases and transcription factors. We found that the Otud7b deficiency attenuated the TCR–CD28–stimulated phosphorylation of several downstream signaling molecules, including Akt, Gsk3β, IKKβ, and MAP kinases in total T cells (Fig. 5 A). The phosphorylation and degradation of IκBα, a target of IKKβ, were also partially inhibited (Fig. 5 A). Consistently, the loss of Otud7b inhibited the activation of three major downstream transcription factors involved in T cell activation, NF-κB, NF-AT, and AP1 (Fig. 5 B). The requirement of Otud7b for the activation of multiple downstream pathways suggested a role for Otud7b in the regulation of TCR-proximal signaling events. Indeed, although the Otud7b deficiency did not appreciably affect Lck phosphorylation, it severely attenuated the phosphorylation of Zap70 (Fig. 5 C). Phosphorylation of the adaptor protein Lat, a major downstream target of Zap70, was also inhibited. These results demonstrate a critical function of Otud7b in mediating TCR–CD28 signaling and suggest its involvement in TCR-proximal signaling steps.

Because the aforementioned experiments were performed using total T cells, we next repeated the signaling studies using purified naive CD4$^+$ and CD8$^+$ T cells to determine whether Otud7b facilitated TCR signaling in both of these T cell subsets in their naive states. Otud7b deficiency inhibited TCR–CD28–stimulated phosphorylation of Zap70, Akt, and Erks in both naive CD4$^+$ T cells (Fig. 5 D) and naive CD8$^+$ T cells (Fig. 5 E). To exclude the possibility of developmental influences, we used a murine T cell line, EL4, which was infected with an Otud7b-specific shRNA for silencing the expression of Otud7b or, alternatively, with a control luciferase shRNA. As seen in primary T cells, Otud7b knockdown by shRNA profoundly inhibited the TCR–CD28–stimulated

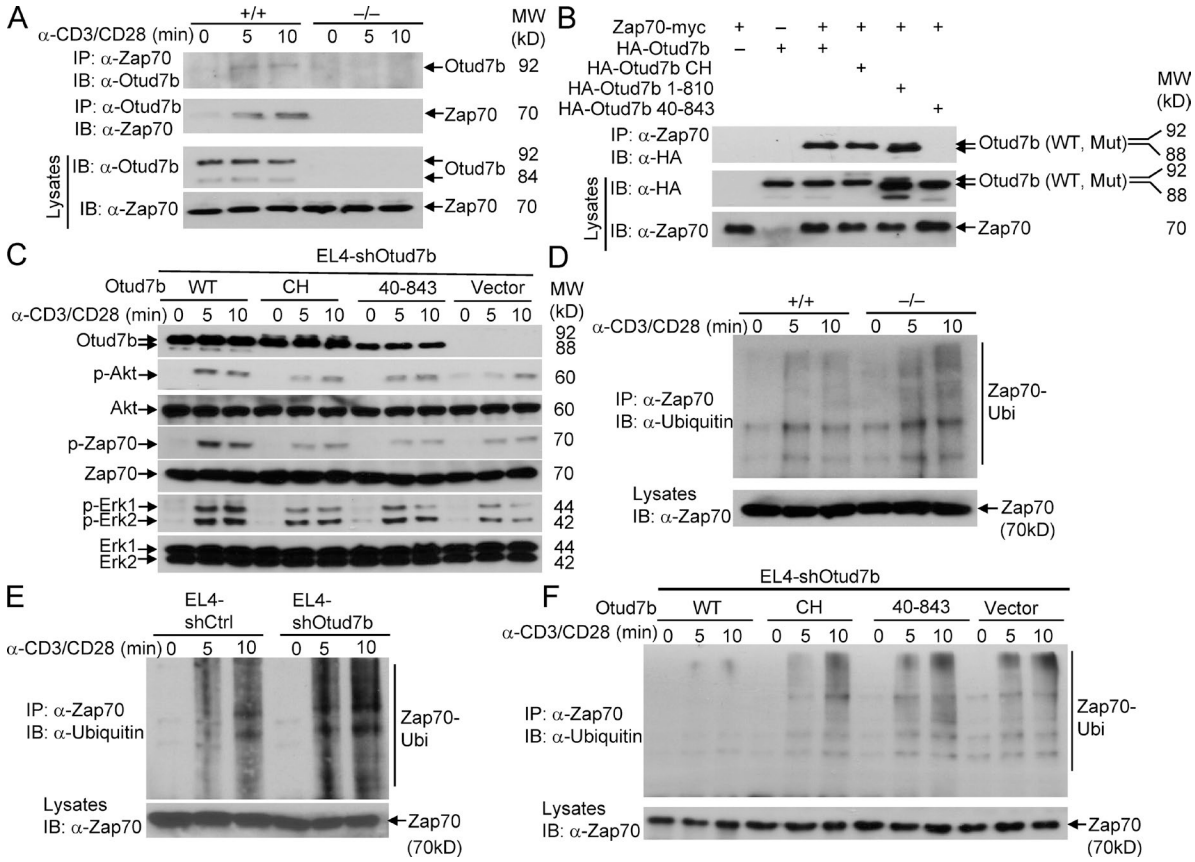

Figure 6. **Otud7b interacts with Zap70 and regulates Zap70 ubiquitination upon TCR stimulation.** (A) Co-IP analysis of Zap70–Otud7b interaction (top two) and direct IB analysis of Zap70 and Otud7b expression (bottom two) in WT or *Otud7b*$^{-/-}$ T cells acutely stimulated with anti-CD3 plus anti-CD28 using an antibody cross-linking method. (B) Co-IP analysis of Zap70–Otud7b interaction (top) and direct IB assays (bottom two) using lysates of HEK293 cells transfected with Zap70, along with expression vectors for Otud7b or its mutants. (C) IB analyses of the indicated phosphorylated (p-) or total proteins in whole-cell lysates of Otud7b-knockdown EL4 cells (EL4-shOtud7b) reconstituted with WT Otud7b or its mutants, stimulated with anti-CD3 plus anti-CD28 antibodies. (D–F) Zap70 ubiquitination assays in anti-CD3/anti-CD28–stimulated primary T cells derived from *Otud7b*$^{+/+}$ or *Otud7b*$^{-/-}$ mice (D), control, or Otud7b-knockdown EL4 cells (E), and Otud7b-knockdown EL4 cells reconstituted with WT Otud7b or its mutants (F). Data are representative of three (A–E) or two (F) independent experiments.

phosphorylation of Zap70, as well as several downstream kinases (Fig. 5 F). Collectively, these data suggest a crucial role for Otud7b in regulating TCR-proximal signaling events in both CD4$^+$ and CD8$^+$ T cells.

Otud7b binds to and deubiquitinates Zap70

We have previously shown that Otud7b deubiqutinates Traf3 and protects Traf3 from ubiquitin-dependent degradation in B cells (Hu et al., 2013). Because Traf3 deficiency also attenuates TCR signaling (Xie et al., 2011), we examined whether Otud7b deficiency promoted the degradation of Traf3 in T cells. However, after extensive biochemical analyses, we did not detect any obvious degradation of Traf3 in *Otud7b*$^{+/+}$ or *Otud7b*$^{-/-}$ T cells along with TCR signaling (unpublished data). This result was consistent with a previous work using Traf3 knockout mice (Xie et al., 2011). Because of the crucial role of Otud7b in regulating the phosphorylation of Zap70, but not Lck, we examined whether Otud7b directly targeted Zap70. Interestingly, although Otud7b did not bind Zap70 in resting T cells, these two proteins formed a complex upon TCR–CD28 stimulation (Fig. 6 A). The Otud7b–Zap70 interaction was further confirmed using a transient transfection experiment in which the overexpressed Otud7b strongly associated with Zap70 (Fig. 6 B). Domain mapping analyses revealed the requirement of the N-terminal UBA domain of Otud7b for its physical interaction with Zap70 (Fig. 6 B), indicating the facilitation of Otud7b–Zap70 interaction by ubiquitination.

To define the function of the UBA domain, as well as the catalytic domain, of Otud7b in regulating Zap70, we reconstituted the *Otud7b*-knockdown EL4 cells with retroviral expression vectors encoding WT human Otud7b or its mutants. Expression of WT Otud7b rescued the defect of the *Otud7b*-knockdown cells in TCR–CD28–stimulated signaling, including phosphorylation of Zap70, Akt and Erk (Fig. 6 C). In contrast, Otud7b mutants harboring point mu-

tations in its catalytic domain, C194S/H358N (CH), or lacking its N-terminal UBA domain (40–843) failed to rescue the TCR signaling events (Fig. 6 C). These results suggest the possibility that Otud7b regulates TCR signaling by physically interacting with and modulating the ubiquitination of Zap70.

We next examined whether Otud7b regulated the ubiquitination of Zap70 along with the induction of TCR signaling. In response to TCR–CD28 ligation, Zap70 became rapidly ubiquitinated in primary T cells, suggesting the coupling of Zap70 activation with its ubiquitination (Fig. 6 D). Importantly, the Otud7b deficiency substantially enhanced the ubiquitination of Zap70 (Fig. 6 D), and the Otud7b knockdown in EL4 cells also enhanced the TCR–CD28–stimulated Zap70 ubiquitination (Fig. 6 E). Furthermore, reconstitution of the *Otud7b*-knockdown EL4 cells with WT Otud7b efficiently suppressed the induction of Zap70 ubiquitination (Fig. 6 F). Consistent with their inability to rescue TCR signaling, the Otud7b mutants Otud7b CH and Otud7b 40–843 failed to suppress the hyperinduction of Zap70 ubiquitination in *Otud7b*-knockdown EL4 cells (Fig. 6 F). Collectively, these results suggest that Otud7b may facilitate TCR signaling by regulating Zap70 ubiquitination.

Otud7b deficiency promotes the association of Zap70 with Sts1/2

In addition to mediating protein degradation, ubiquitin chains play nondegradative roles, including facilitation of protein–protein interactions. Immunoblot (IB) analyses did not detect any appreciable loss of Zap70 along with induction of TCR signaling, and the level of Zap70 was not reduced in the *Otud7b*$^{-/-}$ T cells (Fig. 6 A). Because Otud7b deficiency attenuated Zap70 phosphorylation without affecting activation of its upstream kinase Lck, we hypothesized that the enhanced Zap70 ubiquitination in *Otud7b*$^{-/-}$ T cells might promote the recruitment of a negative regulator to reduce the phosphorylation of Zap70. In this regard, the protein tyrosine phosphoatase Sts1, as well as a homologous and weaker phosphatase, Sts2, have been shown to associate with Zap70 and down-regulate Zap70 phosphorylation and TCR signaling (Carpino et al., 2004, 2009; San Luis et al., 2011). To test our hypothesis, we examined the effect of Otud7b deficiency on the interaction of Zap70 with Sts1/2 upon TCR stimulation. TCR–CD28 ligation triggered rapid association of Zap70 with Sts1 and Sts2 (Fig. 7 A). Remarkably, the signal-induced Zap70–Sts1/2 binding was strongly enhanced in the *Otud7b*$^{-/-}$ T cells (Fig. 7 A). Otud7b knockdown in EL4 cells also enhanced the inducible association of Zap70 with Sts1/2 (Fig. 7 B). This result was specifically caused by loss of Otud7b, as reconstitution of the *Otud7b* knockdown EL4 cells with WT Otud7b efficiently suppressed the TCR–CD28–stimulated Zap70/Sts1 association (Fig. 7 C). Furthermore, consistent with their inability to facilitate TCR signaling (Fig. 6 C), the Otud7b mutants, Otud7b CH and Otud7b 40–843, lacking a functional DUB domain and the UBA domain, respectively, failed to inhibit the recruitment of Sts1 to Zap70 (Fig. 7 C).

The aforementioned results suggested that Otud7b might promote Zap70 phosphorylation by limiting its binding by the phosphatases Sts1/2. To further examine this possibility, we silenced Sts1 in the *Otud7b*-knockdown EL4 cells by a sequential knockdown approach using two different lentiviral shRNA vectors (pLKO.1 and pGIPZ). The Sts1 silencing, using two different shRNAs, in Otud7b-knockdown cells partially restored TCR–CD28–stimulated phosphorylation of Zap70 and downstream kinases (Fig. 7 D). Similarly, Sts1 knockdown in primary T cells rescued the defect of the Otud7b-deficient T cells in TCR signaling (Fig. 7 E). Collectively, these data suggest that the enhanced interaction between Zap70 and Sts1/2 in Otud7b-deficient T cells contributes to attenuated Zap70 activation and TCR signaling.

Zap70 ubiquitination is regulated by Otud7b and involved in Sts1 recruitment

Our finding that the enhanced Zap70–Sts1/2 interaction was associated with Zap70 ubiquitination (Figs. 6 F and 7 C) suggested the possibility that Zap70 ubiquitination might facilitate its association with Sts1/2. To further test this possibility, we first used a mass spectrometry approach to identify the ubiquitination site of Zap70 regulated by Otud7b. We isolated ubiquitinated Zap70, by immunoprecipitation (IP), from murine EL4 cells that were either not treated or stimulated for 5 min with anti-CD3 plus anti-CD28. Mass spectrometry analysis identified K544 as a ubiquitination site of Zap70 in EL4 cells stimulated with anti-CD3 plus anti-CD28 (Fig. 8 A and not depicted). In parallel, we took a transient transfection approach by expressing human Zap70 and ubiquitin in HEK293 cells. Mass spectrometry using this approach also identified K544 as a site of Zap70 ubiquitination, although two other sites (K304 and K538) were also identified (Fig. 8 A and not depicted). When Zap70 was coexpressed with Otud7b, the ubiquitination of Zap70 at both K544 and K538 became undetectable, suggesting that these two sites were sensitive to Otud7b-mediated deubiquitination (Fig. 8 A).

To study the functional significance of Zap70 at the specific lysine residues, we substituted those lysine residues with arginines to generate three Zap70 point mutants, K304R, K538R, and K544R, and examined their ubiquitination and sensitivity to Otud7b. The K304R and K538R mutations had no and a partial effect on Zap70 ubiquitination, respectively, and did not influence the sensitivity of Zap70 to Otud7b-mediated deubiquitination (Fig. 8 B). Interestingly, the K544R mutation substantially interrupted Zap70 ubiquitination, and the residual ubiquitination of this mutant was insensitive to Otud7b (Fig. 8 B). To study the inducible ubiquitination of the Zap70 mutants, we cloned the human WT Zap70 and its K/R mutants into a retroviral vector (pPRI PU-EGFP), generating GFP-Zap70 fusion proteins. We then reconstituted a Zap70-deficient Jurkat T cell line (p116; Williams et al., 1998) using these retroviral expression vectors. As seen with the nontagged Zap70 control, the GFP-Zap70 fusion protein underwent robust ubiquitination in response to

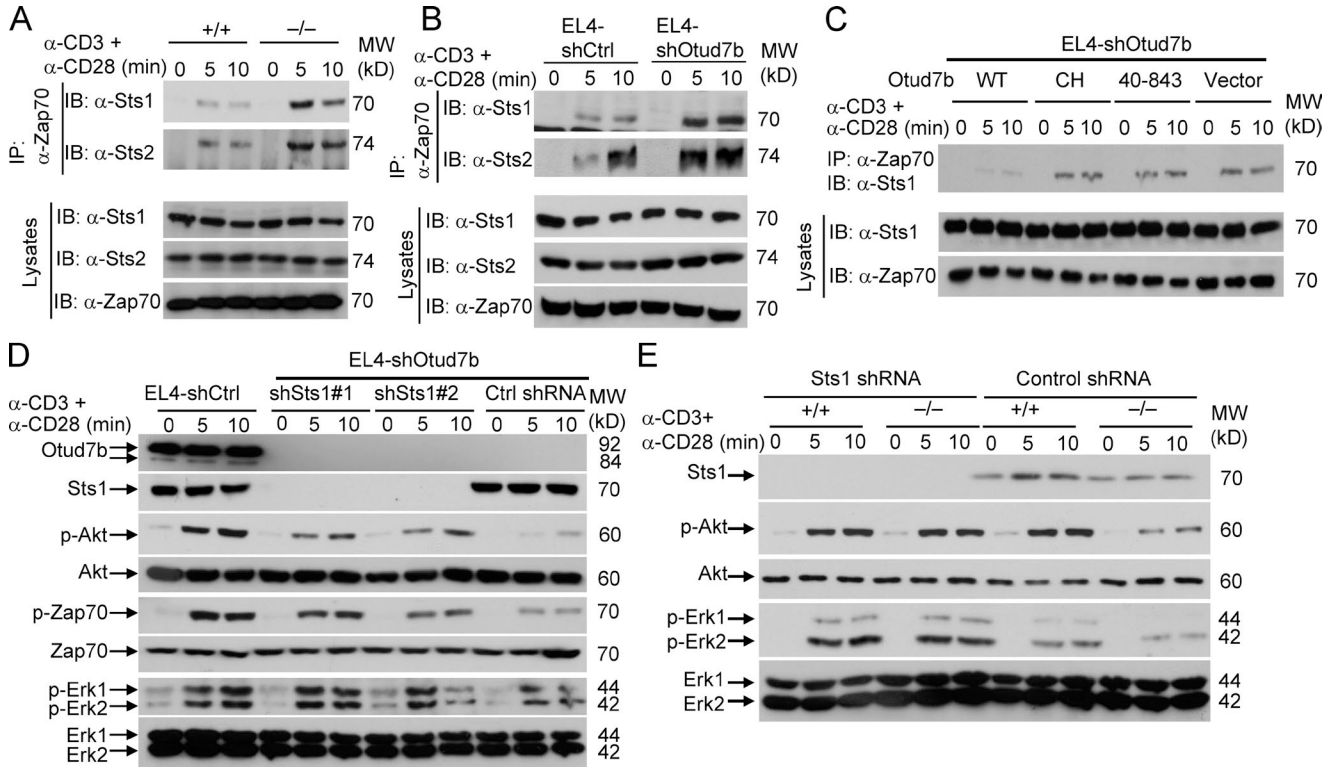

Figure 7. **Otud7b regulates the interaction of Sts1/2 with Zap70.** (A–C) Co-IP assays to detect Zap70–Sts1/2 interaction in whole-cell lysates of anti-CD3/anti-CD28–stimulated *Otud7b*$^{+/+}$ and *Otud7b*$^{-/-}$ primary T cells (A), control and Otud7b-knockdown EL4 cells (B), or Otud7b-knockdown EL4 cells reconstituted with WT Otud7b or its mutants (C). (D) IB analysis of the indicated phosphorylated (p-) and total proteins in whole-cell lysates of control EL4 cells (EL4-shCtrl) or Otud7b-knockdown EL4 cells (EL4-shOtud7b) that were further transduced with a nonsilencing control shRNA (Ctrl shRNA) or two different Sts1 shRNAs (#1 and #2). (E) IB analysis of the indicated phosphorylated (p-) and total proteins in whole-cell lysates of WT or Otud7b$^{-/-}$ T cells transduced with a nonsilencing control shRNA or Sts1 shRNA that were stimulated as indicated. Data are representative of three (A–D) or two (E) independent experiments.

TCR–CD28 stimulation (Fig. 8 C). The K304R and K538R mutants were fully responsive to TCR–CD28–stimulated ubiquitination, whereas the K544R mutant was attenuated in this inducible modification (Fig. 8 C). Importantly, the attenuated ubiquitination of Zap70 K544R mutant was correlated with its reduced ability to recruit Sts1 (Fig. 8 D). This ubiquitination-defective Zap70 mutant was also attenuated in association with Otud7b, consistent with the idea that the Zap70–Otud7b interaction might be facilitated by Zap70 ubiquitination (Fig. 8 D). Furthermore, the p116 cells reconstituted with the K544R Zap70 mutant were hyper-responsive to TCR–CD28–stimulated phosphorylation of Zap70, Akt, and Erk compared with the p116 cells reconstituted with WT Zap70 or K304R and K538R mutants (Fig. 8 E). Collectively, these data suggest that Zap70 ubiquitination at K544 is opposed by the DUB Otud7b and plays a role in recruiting Sts1/2 for Zap70 negative regulation during TCR signaling.

DISCUSSION

The data presented in this paper identified Otud7b as an important regulator of T cell activation that functions as a DUB deubiquitinating Zap70 and, thereby, facilitating activation of TCR-proximal signaling and multiple downstream pathways. Consistently, Otud7b deficiency impaired T cell responses to a bacterial pathogen, *L. monocytogenes*, and also attenuated the T cell–dependent inflammatory responses in two different models, EAE and T cell–induced colitis. Because the role of DUBs in regulating TCR-proximal signaling is a poorly studied topic, our findings provide an example for how deubiquitination of a specific TCR-signaling component contributes to optimal T cell activation and immune responses.

Our data suggest that Otud7b functions as a crucial modulator, although not an essential component, of the TCR signaling complex. Otud7b deficiency attenuated but did not completely block TCR signaling, consistent with partially impaired T cell activation and recall responses. The Otud7b deficiency ameliorated T cell–dependent inflammation in both the EAE and colitis models and caused a predominant defect in the generation of Th1 subset of inflammatory T cells. Although precisely how Otud7b regulates Th1 cell differentiation warrants further studies, it has been shown that the TCR signaling strength plays an important role in regulating CD4^{+}

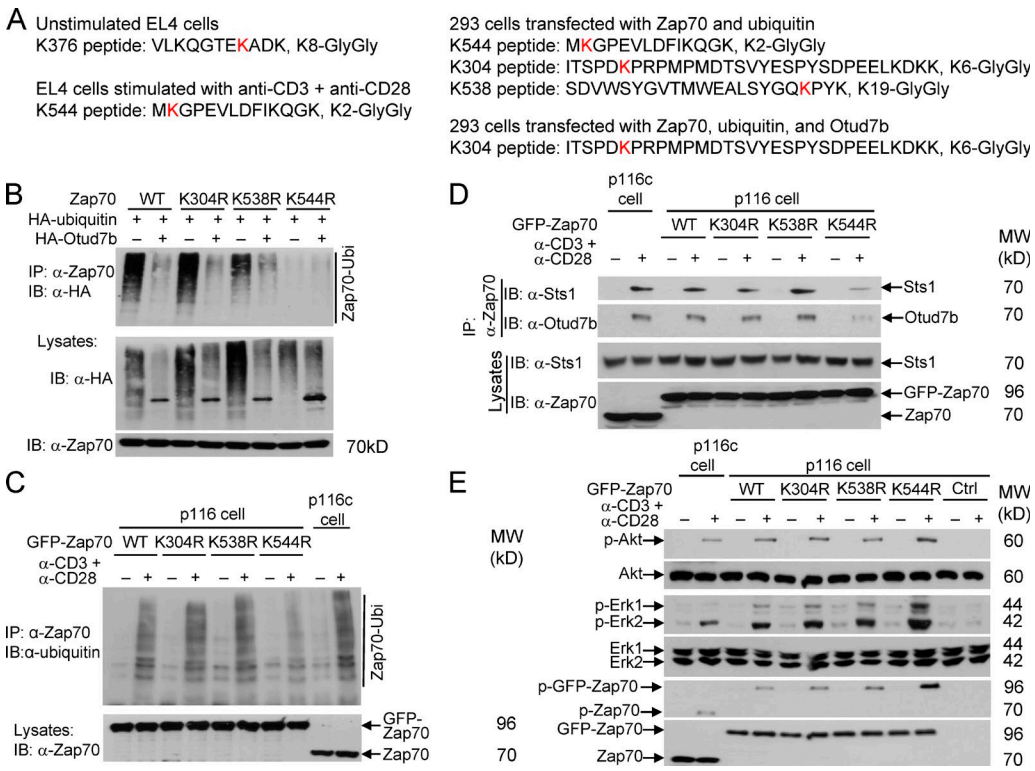

Figure 8. **Zap70 ubiquitination at K544 mediates recruitment of Sts1/2.** (A) Results of mass spectrometry analyses of Zap70 ubiquitination in EL4 cells stimulated for 5 min with anti-CD3 plus anti-CD28 (A) or 293 cells transfected with Zap70 and ubiquitin (B–D), showing the identified peptides and the ubiquitination sites (K544, K304, and K538) highlighted in red. (B) Zap70 ubiquitination assay using lysates of 293 cells transfected with WT Zap70 or its indicated mutants along with HA-tagged ubiquitin, either in the presence (+) or absence (–) of Otud7b. (C) Zap70 ubiquitination assay using lysates of Zap70-deficient Jurkat cell line (p116) reconstituted with WT or mutant forms of Zap70 fused to GFP, which were either not treated (–) or stimulated (+) for 10 min with anti-CD3 plus anti-CD28 antibodies. The p116 cells reconstituted with untagged Zap70 (p116c) was included as a positive control. (D) Co-IP assays to detect the Zap70/Sts1 binding in the p116 cells described in C. (E) IB analysis of the phosphorylated (p-) and total proteins in the p116 cells described in C. Data are representative of three independent experiments (B–E).

T cell differentiation (Zhu et al., 2010; Tubo and Jenkins, 2014; van Panhuys et al., 2014). Although weak signals are sufficient for the induction of Th2 cell differentiation, Th1 cell differentiation requires strong TCR signaling. Thus, it is likely that compromised TCR signaling may contribute to the impaired Th1 differentiation of Otud7b-deficient $CD4^+$ T cells.

Ubiquitination plays an important role in the regulation of T cell activation and tolerance (Park et al., 2014). Several E3 ubiquitin ligases, such as Cbl-b, GRAIL, and ITCH, are known to negatively regulate TCR signaling by targeting TCR-signaling molecules for ubiquitin-dependent degradation (Park et al., 2014). Nondegradative ubiquitination has also emerged as a mechanism that regulates TCR-proximal signaling. For example, Itch and Cbl-b act cooperatively to conjugate K33-linked ubiquitin chains to TCRζ chain and inhibit its signaling function (Huang et al., 2010). Our current work established Otud7b as a DUB that inhibits ubiquitination of Zap70. In response to TCR signaling, Otud7b was rapidly recruited to Zap70 via a mechanism that required the UBA domain of Otud7b, suggesting the possibility that Otud7b interacted with ubiquitinated Zap70. We further showed that both of the UBA domain and the DUB catalytic activity of Otud7b were required for inhibiting Zap70 ubiquitination and facilitating Zap70 phosphorylation and TCR signaling, suggesting that Otud7b functions as a specific DUB of Zap70 in the TCR pathway.

Our data suggest that Otud7b regulated nondegradative ubiquitination of Zap70, as we were unable to detect a difference in the fate of Zap70 between the WT and Otud7b-deficient T cells under homeostatic or TCR-stimulated conditions. The fact that the effect of Otud7b deficiency on Zap70 phosphorylation was seen within a very short time (5 min) of TCR stimulation also suggested a nondegradative mechanism of regulation. The Otud7b-mediated Zap70 deubiquitination appears to serve as a mechanism to prevent the association of Zap70 by negative-regulatory phosphatases, Sts1 and Sts2. We found that the accumulation of ubiquitinated Zap70 in Otud7b-deficient T cells was associated with enhanced recruitment of Sts1/2 to Zap70. Our proteomic studies identified K544 as a ubiquitination acceptor site of Zap70 that is regulated by Otud7b, and mutation of this site generated a Zap70 mutant (K544R) that was defective in both

ubiquitination and Sts1/2 binding and displaying aberrantly elevated signaling activity. Sts1 knockdown in Otud7b-deficient T cells partially restored TCR-stimulated Zap70 phosphorylation and downstream signaling events, likely due to functional redundancy of Sts1 and Sts2. Based on these findings, we propose a model, in which Otud7b facilitates Zap70 activation and TCR-proximal signaling by deubiquitinating Zap70 and limiting its binding by the negative regulator Sts1.

Our data are in agreement with a recent study that Zap70 ubiquitination serves as a mechanism to recruit Sts1 and Sts2 for Zap70 negative regulation (Yang et al., 2015). This recent study identified Nrdp1 as an E3 ubiquitin ligase that mediates K33 ubiquitination of Zap70 in $CD8^+$ T cells (Yang et al., 2015). Nrdp1-mediated Zap70 ubiquitination promotes Zap70 dephosphorylation by Sts1 and Sts2, thereby negatively regulating Zap70 phosphorylation and TCR-proximal signaling. Interestingly, under transient transfection conditions, Nrdp1 induces Zap70 ubiquitination at six different K residues, and mutation of K538 blocks Nrdp1-induced Zap70 ubiquitination and promotes the signaling function of Zap70. Because Nrdp1 does not regulate TCR signaling in $CD4^+$ T cells, additional E3 ubiquitin ligases likely regulate the ubiquitination and signaling function of Zap70 (Yang et al., 2015). This possibility is further suggested by our present finding that Otud7b deficiency promotes Zap70 ubiquitination and inhibits Zap70 phosphorylation in both $CD4^+$ and $CD8^+$ T cells. Otud7b likely opposes the action of a Zap70 E3 ligase other than Nrdp1, because Otud7b inhibits Zap70 ubiquitination mainly at K544, which differs from the Nrdp1 target site (K538). Together, these results suggest that the signaling function of Zap70 may be regulated through its ubiquitination at different K residues.

We have previously shown that Otud7b inhibits the ubiquitination and inducible degradation of Traf3 in B cells, thereby negatively regulating activation of the nocanonical NF-κB by TNF receptor family members, such as BAFF receptor and CD40 (Hu et al., 2013). Notably, like Otud7b, Traf3 plays a positive role in regulating TCR-proximal signaling, and Traf3 ablation in T cells attenuates TCR proximal signaling and impairs T cell activation (Xie et al., 2011). Precisely how Traf3 facilitates TCR signaling has remained unknown, but Traf3 has been shown to be recruited to the TCR–CD28 signaling complex (Xie et al., 2011). We found that Traf3 physically interacted with Otud7b in T cells (unpublished data), although we could not detect appreciable degradation of Traf3 along with TCR–CD28 ligation in either WT or Otud7b-deficient T cells. Whether the Otud7b/Traf3 interplay is also partially involved in the regulation of T cell activation requires further study. Nevertheless, our data suggest Zap70 to be the major target of Otud7b in the TCR pathway. These findings provide an example for how T cell activation and T cell–mediated immune responses are regulated via deubiquitination of a specific signaling molecule of the TCR pathway. Because Otud7b deficiency does not completely block T cell activation but rather predominantly impairs the inflammatory T cell responses, our findings also implicate Otud7b as a potential drug target for manipulation of immune and inflammatory reactions.

MATERIALS AND METHODS

Mice. Mice with germline *Otud7b* deletion ($Otud7b^{-/-}$ mice; in C57BL/6 × 129/sv genetic background) were generated by a conventional gene-targeting approach (Deltagen, Inc.) and backcrossed for 10 generations to the C57BL/6 background (Hu et al., 2013). $Otud7b^{+/-}$ heterozygous mice were bred to generate age-matched $Otud7b^{+/+}$ and $Otud7b^{-/-}$ mice for experiments. Mice deficient in recombination-activating gene 1 ($Rag1^{-/-}$) and lacking T and B cells, and mice deficient in both *Tcrb* and *Tcrd* genes ($Tcrb^{-/-}Tcrd^{-/-}$) and lacking T cells were obtained from The Jackson Laboratory (in C57BL/6 background). Outcomes of animal experiments were collected blindly and recorded based on ear-tag numbers of the experimental mice. Mice were maintained in specific pathogen–free facility, and all animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center.

Plasmids. Hemagglutinin (HA)-tagged Otud7b was cloned into the retroviral vector pCLXSN(GFP), and its catalytically inactive mutant, C194S/H358R (CH), and truncation mutants (1–810 and 40–843) were created as previously described (Hu et al., 2013). Otud7b shRNA and a control luciferase shRNA in pLKO.1 lentiviral vector were obtained from Sigma-Aldrich; Sts1 shRNAs and a nonsilencing control shRNA in pGIPZ lentiviral vector were purchased from Thermo Fisher Scientific. The expression vectors encoding HA-ubiquitin was cloned into pcDNA vector as previously described (Xiao et al., 2001), and Myc-tagged human Zap70 in pSXSR vector (pSXSR-Myc-hZap70) was obtained from L.E. Samelson (National Cancer Institute, National Institutes of Health, Bethesda, MD). Zap70 mutants harboring K-to-R substitutions (K304R, K538R, and K544R) were generated by site-directed mutagenesis using the pSXSR-Myc-Zap70 template, and the mutations were confirmed by sequencing. The WT and mutant forms of Zap70 were subcloned into the retroviral vector pPRIPU-eGFP for expressing GFP-fusion proteins (provided by P. Martin, Institut Gustave Roussy, Villejuif, France; Albagli-Curiel et al., 2007).

Antibodies and reagents. Antibodies for Akt1 (B-1), HSP60 (H1), Zap70 (1E7.2), p38 (H-147), Jnk1 (C-19), IKKβ (H470), GSK3β (H76), Lck(3A5), Lat (FL233), Traf3 (H-122), ubiquitin (PD-1), Erk (K-23), and phospho-Erk (E-4) were obtained from Santa Cruz Biotechnology, Inc. Horseradish peroxidase–conjugated anti-HA antibody (3F10) was purchased from Roche. Antibodies for phospho-Akt (Ser473; D9E), phospho-p38 (9211), phospho-IκBα (9241), phospho-Lck (2751), phospho-GSK3β (9331), phospho-Jnk (9251), phospho-Lat (3584), phospho-Syc family kinase

(2101), and phospho-Zap70 (Tyr319; 2701) were obtained from Cell Signaling Technology. Anti-Otud7b was purchased from Proteintech, and anti–b-actin mouse monoclonal antibody was purchased from Sigma-Aldrich. Anti-Sts1 (ab34781) and anti-Sts2 (ab105058) antibodies were obtained from Abcam. Functional grade monoclonal antibodies for murine CD3 (145-2C11) and CD28 (37.51), and for human CD3 (OKT3; 16–0037-85) and CD28 (16–0289-85) were obtained from eBioscience. Cross-linking anti-Ig antibodies (goat anti–hamster and goat anti–mouse) were purchased from Southern Biotech. Fluorescence-labeled antibodies for CD4 (RM4-5), CD8 (53–6.7), CD44 (IM7), CD45RB (C363.16A), CD62L (MEL-14), IFN-γ (XMG1.2), IL-4 (11B11), IL-17A (ebio17B7), and Foxp3 (FJK-16s) were purchased from eBioscience. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were obtained from Sigma-Aldrich.

T cell isolation and stimulation. Primary T cells were isolated from the spleen and LNs of young adult mice (6–8 wk old) using anti-CD90.2 magnetic beads. Naive $CD4^+$ and $CD8^+$ T cells were further purified by flow cytometric cell sorting based on $CD4^+CD25^-CD44^{lo}CD62L^{hi}$ and $CD8^+CD44^{lo}CD62L^{hi}$ surface markers, respectively. The cells were stimulated with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) in replicate wells of 96-well plates (10^5 cells per well) for ELISA, 12-well plates (10^6 cells per well) for quantitative RT-PCR (qRT-PCR), and 6-well plates (5×10^6 per well) for IB assays. Where indicated, the cells were acutely stimulated using an antibody cross-linking protocol as previously described (Reiley et al., 2007).

$CD4^+$ T cell differentiation. Naive $CD4^+$ T cells ($CD4^+CD25^-CD44^{lo}CD62L^{hi}$) were isolated from spleens and LNs of WT or *Otud7b*$^{-/-}$ mice and stimulated with plate-coated anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) under Th1 (10 ng/ml IL-12 and 5 μg/ml anti-IL-4), TGF-β Th17 (1 ng/ml TGF-β, 10 ng/ml IL-6, 5 μg/ml anti-IFN-γ, and 5 μg/ml anti–IL-4), IL-1β Th17 (10 ng/ml IL-1β, 10 ng/ml IL-6, 20 ng/ml IL-23, 5 μg/ml anti–IFN-γ, and 5 μg/ml anti–IL-4), or T reg cell (1 ng/ml TGF-β and 10 ng/ml IL-2) conditions. After 3 d of differentiation, cells were subjected to intracellular cytokine staining (ICS) and flow cytometry analyses.

Flow cytometry, cell sorting, and ICS. Suspensions of splenocytes and LN cells were subjected to flow cytometry and cell sorting as previously described (Reiley et al., 2006) using LSR II (BD) and FACSAria (BD) flow cytometers, respectively. For ICS, T cells were stimulated for 4 h with PMA plus ionomycin in the presence of a protein transport inhibitor, monensin (1:1,000; 00–4505-51; eBioscience), and then subjected to intracellular staining of different cytokines and flow cytometry analyses. For analyzing in vivo–primed, antigen-specific T cells, the T cells were stimulated in vitro with the indicated antigenic peptides in the presence of monensin and then subjected to ICS. The data were analyzed using FlowJo software (Tree Star).

ELISA and qRT-PCR. Supernatants of in vitro cell cultures were analyzed by ELISA using a commercial assay system (eBioscience). Total RNA was prepared from the indicated cells and subjected to qRT-PCR using the following gene-specific primers (all for mouse genes): *Il2* forward, 5′-CCTGAGCAGGATGGAGAATTACA-3′; *Il2* reverse, 5′-TCCAGAACATGCCGCAGAG-3′; *Ifng* forward, 5′-CAGCAACAGCAAGGCGAAA-3′; *Ifng* reverse, 5′-CTGGACCTGTGGGTTGTTGAC-3′; *Actb* forward, 5′-CGTGAAAAGATGACCCAGATCA-3′; and *Actb* reverse, 5′-CACAGCCTGGATGGCTACGT-3′.

IB, co-IP, ubiquitination assays, and EMSA. Total cell lysates were prepared and subjected to IB and co-IP assays as previously described (Waterfield et al., 2003; Reiley et al., 2007). For ubiquitination assays, cells were lysed in RIPA buffer with 1% SDS and boiled for 5 min. After being diluted for 10 times with RIPA buffer lacking SDS, cell lysates were subjected to IP by anti-Zap70 antibody. The ubiquitinated Zap70 were detected by IB using an anti-ubiquitin antibody. IB images were quantified by the ImageJ program, and data were presented as ratios between phosphorylated proteins and corresponding total proteins (for phospho-IB), between co-precipitated proteins and corresponding input proteins (for co-IP), and between specific ubiquitinated proteins and total cellular ubiquitinated proteins (for ubiquitination assay). In some cases, data were presented as arbitrary units.

EMSAs were performed using nuclear extracts and [^{32}P]-radiolabeled oligonucleotide probes for NF-κB (5′-CAACGGCAGGGGAATTCCCCTCTCCTT-3′), NF-AT (5′-TCGAGGAGGAAAAACTGTTTCATA-3′), AP-1 (5′-GATCTAGTGATGAGTCAGCCG-3′), or the control transcription factor NF-Y (5′-AAGAGATTAACCAATCACGTACGGTCT-3′).

Mass spectrometry analysis of Zap70 ubiquitination sites. EL4 T cells were stimulated for 5 min with anti-CD3 plus anti-CD28, and endogenous Zap70 was isolated by IP using anti-Zap70 (1E7.2). 293 cells were transfected with pSXSR-Myc-hZap70 and ubiquitin, with or without pCLXSN-HA-Otud7b, and the transfected Zap70 was isolated by IP using anti-Zap70 antibody. The conditions of cell lysate preparation and IP were the same as described for ubiquitination assays. To identify the ubiquitination sites of Zap70, the isolated Zap70 proteins were subjected to mass spectrometry analysis in the Pathway Discovery Mass Spectrometry/Identification of Protein Complex Core Facility at Baylor College of Medicine (Waco, TX).

In brief, the washed IP beads were boiled in 30 μl of 1X NUPAGE LDS sample buffer (Invitrogen) and subjected to SDS-PAGE (NuPAGE 10% Bis-Tris Gel; Invitrogen). The eluted proteins were visualized with Coomassie Brilliant blue

stain and excised into six gel pieces according to molecular size. The individual gel pieces were destained and subject to in-gel digestion using trypsin (GenDepot T9600). The tryptic peptides were resuspended in 10 µl of loading solution (5% methanol containing 0.1% formic acid) and subjected to nanoflow LC-MS/MS analysis with a nano-LC 1000 system (Thermo Fisher Scientific) coupled to LTQ Orbitrap Elite (Thermo Fisher Scientific) mass spectrometer. The peptides were loaded onto a Reprosil-Pur Basic C18 (1.9 µm; Dr. Maisch GmbH) precolumn of 2 cm × 100 µm size. The precolumn was switched in-line with an in-housed 50 mm × 150 µm analytical column packed with Reprosil-Pur Basic C18 equilibrated in 0.1% formic acid/water. The peptides were eluted using a 75-min discontinuous gradient of 4–26% acetonitrile/0.1% formic acid at a flow rate of 600 nl/min. The eluted peptides were directly electro-sprayed into LTQ Orbitrap Elite mass spectrometer operated in the data-dependent acquisition mode acquiring fragmentation spectra of the top 50 strongest ions.

Obtained MS/MS spectra of EL4 Zap70 and transfected human Zap70 were searched against target-decoy mouse and human refseq database, respectively, in Proteome Discoverer 1.4 interface (Thermo Fisher Scientific) with Mascot algorithm (Mascot 2.4; Matrix Science). The precursor mass tolerance was confined within 20 ppm with fragment mass tolerance of 0.5 D and a maximum of two missed cleavages allowed. Dynamic modification of Oxidation, protein N-terminal Acetylation, Ubiquitination (diglycine), and Destreak were allowed. The peptides identified from mascot result file were validated with 5% false discover rate and subject to manual verifications. iBAQ algorithm was used to calculate protein abundance to compare relative amount between different proteins in the sample. Simply, iBAQ was calculated based on normalization of summed peptide intensity divided by the number of theoretically observable tryptic peptides of certain protein.

Cell culture, gene silencing, and overexpression. For gene silencing, lentiviral particles were prepared by transfecting HEK293 cells with pLKO.1 or pGIPZ lentiviral vectors encoding specific shRNAs or control shRNAs along with packaging plasmids. The packaged viruses were then used to infect the indicated cells, followed by selection of the infected cells by puromycin (1 µg/ml) for 7 d (for the pLKO.1 vector) or by sorting GFP^+ cells (for the pGIPZ vector, which carries the GFP gene). To knock down Sts1 in the primary T cells, $CD4^+$ T cells were isolated from WT and Otud7b-deficient mice by flow cytometric cell sorting and cultured with plate-coated anti-CD3 antibody plus anti-CD28 antibody for 24 h, before being infected by pGIPz-based control shRNA or Sts1 shRNA and cultured for another 48 h. The infected cells were sorted based on GFP expression and starved overnight before being restimulated by cross-linking anti-CD3 plus anti-CD28 antibodies to examine the TCR signaling.

Zap70-deficient Jurkat cell line p116 and the Zap70-recostituted p116 control line (p116c) were provided by L.P. Kane (University of Pittsburgh, Pittsburgh, PA). The p116 cells were reconstituted with WT or mutant forms of Zap70 by retroviral infection.

EAE induction and assessment. Active EAE was induced essentially as previously described (Jin et al., 2009), except the mice received only a single MOG_{35-55} peptide immunization. Mice were monitored daily for the disease symptoms. For the MOG-specific T cell recall response, splenocytes and draining LN cells were isolated from MOG-immunized mice on day 10 of immunization and stimulated in vitro with different doses of MOG_{35-55} peptide for 48 h. Cell proliferation was measured based $[^3H]$-thymidine incorporation, and supernatants of cell culture were subjected to ELISA.

Bone marrow chimeras. T cell–deficient *TCRβ/δ*$^{-/-}$ mice were lethally irradiated (950 rad) and adoptively transferred with WT or *Otud7b*$^{-/-}$ bone marrows mixed with TCRβ/δ$^{-/-}$ bone marrows (1:3) to generate WT and *Otud7b*$^{-/-}$ chimeric mice, respectively. After 6 wk, the WT and KO chimeric mice were immunized for EAE induction.

T cell adoptive transfer model of colitis. $CD4^+CD25^-CD45RB^{hi}$ cells from WT and *Otud7b*$^{-/-}$ mice were prepared by FACS sorting and adoptively transferred (via i.p. injection) into *Rag1*$^{-/-}$ mice (4×10^5 cells/mouse). Recipient mice were observed daily, and bodyweight was measured weekly. At the end of the experiment (8 wk), all mice were sacrificed, and intestines were removed for hematoxylin and eosin (H&E) staining and histology analysis.

***L. monocytogenes* infection.** Recombinant *L. monocytogenes* expressing a truncated OVA protein (aa 134–387; LM-OVA) was provided by H. Shen (University of Pennsylvania, Philadelphia, PA). Age- and gender-matched WT and *Otud7b*$^{-/-}$ mice (7–8 wk old) were infected i.v. with LM-OVA (10^5 CFU per mouse) and sacrificed on day 7 after infection. Splenocytes were restimulated with the MHC class II–restricted $LLO_{190-201}$ peptide NEKYAQAYPNVS (15 µg/ml) or the MHC class I–restricted $OVA_{257-264}$ peptide SII NFEKL (15 µg/ml) for 5 h, followed by treatment with monesin for 1 h. Intracellular IFN-γ expression was analyzed by ICS and flow cytometry.

Statistical analysis. Statistical analysis was performed using Prism software. Two-way ANOVA followed by Bonferroni's multiple comparisons test was used to compare differences between multiple groups. Two-tailed unpaired Student's *t* tests were performed and p-values <0.05 were considered significant. The level of significance is indicated as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. In the animal studies, four mice are required for each group based on the calculation to achieve a 2.3-fold change (effect size) in two-tailed Student's *t* test with 90% power and a significance level of 5%. All statistical tests are justified as appropriate, and data meet the assumptions of

the tests. The variance is similar between the groups being statistically compared.

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REFERENCES

Albagli-Curiel, O., Y. Lécluse, P. Pognonec, K.E. Boulukos, and P. Martin. 2007. A new generation of pPRIG-based retroviral vectors. *BMC Biotechnol.* 7:85. http://dx.doi.org/10.1186/1472-6750-7-85

Carpino, N., S. Turner, D. Mekala, Y. Takahashi, H. Zang, T.L. Geiger, P. Doherty, and J.N. Ihle. 2004. Regulation of ZAP-70 activation and TCR signaling by two related proteins, Sts-1 and Sts-2. *Immunity.* 20:37–46. http://dx.doi.org/10.1016/S1074-7613(03)00351-0

Carpino, N., Y. Chen, N. Nassar, and H.W. Oh. 2009. The Sts proteins target tyrosine phosphorylated, ubiquitinated proteins within TCR signaling pathways. *Mol. Immunol.* 46:3224–3231. http://dx.doi.org/10.1016/j.molimm.2009.08.015

El-behi, M., A. Rostami, and B. Ciric. 2010. Current views on the roles of Th1 and Th17 cells in experimental autoimmune encephalomyelitis. *J. Neuroimmune Pharmacol.* 5:189–197. http://dx.doi.org/10.1007/s11481-009-9188-9

Foulds, K.E., L.A. Zenewicz, D.J. Shedlock, J. Jiang, A.E. Troy, and H. Shen. 2002. Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses. *J. Immunol.* 168:1528–1532. http://dx.doi.org/10.4049/jimmunol.168.4.1528

Gardam, S., F. Sierro, A. Basten, F. Mackay, and R. Brink. 2008. TRAF2 and TRAF3 signal adapters act cooperatively to control the maturation and survival signals delivered to B cells by the BAFF receptor. *Immunity.* 28:391–401. http://dx.doi.org/10.1016/j.immuni.2008.01.009

Heissmeyer, V., F. Macián, S.H. Im, R. Varma, S. Feske, K. Venuprasad, H. Gu, Y.C. Liu, M.L. Dustin, and A. Rao. 2004. Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nat. Immunol.* 5:255–265. http://dx.doi.org/10.1038/ni1047

Hu, H., G.C. Brittain, J.H. Chang, N. Puebla-Osorio, J. Jin, A. Zal, Y. Xiao, X. Cheng, M. Chang, Y.X. Fu, et al. 2013. OTUD7B controls non-canonical NF-κB activation through deubiquitination of TRAF3. *Nature.* 494:371–374. http://dx.doi.org/10.1038/nature11831

Huang, F., and H. Gu. 2008. Negative regulation of lymphocyte development and function by the Cbl family of proteins. *Immunol. Rev.* 224:229–238. http://dx.doi.org/10.1111/j.1600-065X.2008.00655.x

Huang, H., M.S. Jeon, L. Liao, C. Yang, C. Elly, J.R. Yates III, and Y.C. Liu. 2010. K33-linked polyubiquitination of T cell receptor-ζ regulates proteolysis-independent T cell signaling. *Immunity.* 33:60–70. http://dx.doi.org/10.1016/j.immuni.2010.07.002

Jameson, S.C. 2005. T cell homeostasis: keeping useful T cells alive and live T cells useful. *Semin. Immunol.* 17:231–237. http://dx.doi.org/10.1016/j.smim.2005.02.003

Jeon, M.-S., A. Atfield, K. Venuprasad, C. Krawczyk, R. Sarao, C. Elly, C. Yang, S. Arya, K. Bachmaier, L. Su, et al. 2004. Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction. *Immunity.* 21:167–177. http://dx.doi.org/10.1016/j.immuni.2004.07.013

Jin, W., X.F. Zhou, J. Yu, X. Cheng, and S.C. Sun. 2009. Regulation of Th17 cell differentiation and EAE induction by MAP3K NIK. *Blood.* 113:6603–6610. http://dx.doi.org/10.1182/blood-2008-12-192914

Lara-Tejero, M., and E.G. Pamer. 2004. T cell responses to *Listeria monocytogenes*. *Curr. Opin. Microbiol.* 7:45–50. http://dx.doi.org/10.1016/j.mib.2003.12.002

Liu, Y.C., J. Penninger, and M. Karin. 2005. Immunity by ubiquitylation: a reversible process of modification. *Nat. Rev. Immunol.* 5:941–952. http://dx.doi.org/10.1038/nri1731

Morrissey, P.J., K. Charrier, S. Braddy, D. Liggitt, and J.D. Watson. 1993. CD4+ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified $CD4^+$ T cells. *J. Exp. Med.* 178:237–244. http://dx.doi.org/10.1084/jem.178.1.237

Ohashi, P.S. 2002. T-cell signalling and autoimmunity: molecular mechanisms of disease. *Nat. Rev. Immunol.* 2:427–438.

Park, Y., H.S. Jin, D. Aki, J. Lee, and Y.C. Liu. 2014. The ubiquitin system in immune regulation. *Adv. Immunol.* 124:17–66. http://dx.doi.org/10.1016/B978-0-12-800147-9.00002-9

Reiley, W.W., M. Zhang, W. Jin, M. Losiewicz, K.B. Donohue, C.C. Norbury, and S.C. Sun. 2006. Regulation of T cell development by the deubiquitinating enzyme CYLD. *Nat. Immunol.* 7:411–417. http://dx.doi.org/10.1038/ni1315

Reiley, W.W., W. Jin, A.J. Lee, A. Wright, X. Wu, E.F. Tewalt, T.O. Leonard, C.C. Norbury, L. Fitzpatrick, M. Zhang, and S.C. Sun. 2007. Deubiquitinating enzyme CYLD negatively regulates the ubiquitin-dependent kinase Tak1 and prevents abnormal T cell responses. *J. Exp. Med.* 204:1475–1485. http://dx.doi.org/10.1084/jem.20062694

Reyes-Turcu, F.E., K.H. Ventii, and K.D. Wilkinson. 2009. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. *Annu. Rev. Biochem.* 78:363–397. http://dx.doi.org/10.1146/annurev.biochem.78.082307.091526

Robinson, A.P., C.T. Harp, A. Noronha, and S.D. Miller. 2014. The experimental autoimmune encephalomyelitis (EAE) model of MS: utility for understanding disease pathophysiology and treatment. *Handb. Clin. Neurol.* 122:173–189. http://dx.doi.org/10.1016/B978-0-444-52001-2.00008-X

San Luis, B., B. Sondgeroth, N. Nassar, and N. Carpino. 2011. Sts-2 is a phosphatase that negatively regulates zeta-associated protein (ZAP)-70 and T cell receptor signaling pathways. *J. Biol. Chem.* 286:15943–15954. http://dx.doi.org/10.1074/jbc.M110.177634

Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan. 2009. T cell activation. *Annu. Rev. Immunol.* 27:591–619. http://dx.doi.org/10.1146/annurev.immunol.021908.132706

Sprent, J., and C.D. Surh. 2011. Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells. *Nat. Immunol.* 12:478–484. http://dx.doi.org/10.1038/ni.2018

Sun, S.C. 2008. Deubiquitylation and regulation of the immune response. *Nat. Rev. Immunol.* 8:501–511. http://dx.doi.org/10.1038/nri2337

Takada, K., and S.C. Jameson. 2009. Naive T cell homeostasis: from awareness of space to a sense of place. *Nat. Rev. Immunol.* 9:823–832. http://dx.doi.org/10.1038/nri2657

Tubo, N.J., and M.K. Jenkins. 2014. TCR signal quantity and quality in $CD4^+$ T cell differentiation. *Trends Immunol.* 35:591–596. http://dx.doi.org/10.1016/j.it.2014.09.008

van Panhuys, N., F. Klauschen, and R.N. Germain. 2014. T-cell-receptor-dependent signal intensity dominantly controls $CD4^+$ T cell polarization in vivo. *Immunity.* 41:63–74. http://dx.doi.org/10.1016/j.immuni.2014.06.003

Wakil, A.E., Z.E. Wang, J.C. Ryan, D.J. Fowell, and R.M. Locksley. 1998. Interferon γ derived from $CD4^+$ T cells is sufficient to mediate T helper cell type 1 development. *J. Exp. Med.* 188:1651–1656. http://dx.doi.org/10.1084/jem.188.9.1651

Waterfield, M.R., M. Zhang, L.P. Norman, and S.C. Sun. 2003. NF-κB1/p105 regulates lipopolysaccharide-stimulated MAP kinase signaling by governing the stability and function of the Tpl2 kinase. *Mol. Cell.* 11:685–694. http://dx.doi.org/10.1016/S1097-2765(03)00070-4

Williams, B.L., K.L. Schreiber, W. Zhang, R.L. Wange, L.E. Samelson, P.J. Leibson, and R.T. Abraham. 1998. Genetic evidence for differential coupling of Syk family kinases to the T-cell receptor: reconstitution studies in a ZAP-70-deficient Jurkat T-cell line. *Mol. Cell. Biol.* 18:1388–1399. http://dx.doi.org/10.1128/MCB.18.3.1388

Xiao, G., M.E. Cvijic, A. Fong, E.W. Harhaj, M.T. Uhlik, M. Waterfield, and S.C. Sun. 2001. Retroviral oncoprotein Tax induces processing of NF-κB2/p100 in T cells: evidence for the involvement of IKKα. *EMBO J.* 20:6805–6815. http://dx.doi.org/10.1093/emboj/20.23.6805

Xie, P., L.L. Stunz, K.D. Larison, B. Yang, and G.A. Bishop. 2007. Tumor necrosis factor receptor-associated factor 3 is a critical regulator of B cell homeostasis in secondary lymphoid organs. *Immunity.* 27:253–267. http://dx.doi.org/10.1016/j.immuni.2007.07.012

Xie, P., Z.J. Kraus, L.L. Stunz, Y. Liu, and G.A. Bishop. 2011. TNF receptor-associated factor 3 is required for T cell-mediated immunity and TCR/CD28 signaling. *J. Immunol.* 186:143–155. http://dx.doi.org/10.4049/jimmunol.1000290

Yang, M., T. Chen, X. Li, Z. Yu, S. Tang, C. Wang, Y. Gu, Y. Liu, S. Xu, W. Li, et al. 2015. K33-linked polyubiquitination of Zap70 by Nrdp1 controls $CD8^+$ T cell activation. *Nat. Immunol.* 16:1253–1262. http://dx.doi.org/10.1038/ni.3258

Zepp, J., L. Wu, and X. Li. 2011. IL-17 receptor signaling and T helper 17-mediated autoimmune demyelinating disease. *Trends Immunol.* 32:232–239. http://dx.doi.org/10.1016/j.it.2011.02.007

Zhu, J., H. Yamane, and W.E. Paul. 2010. Differentiation of effector CD4 T cell populations (*). *Annu. Rev. Immunol.* 28:445–489. http://dx.doi.org/10.1146/annurev-immunol-030409-101212