The Ube2m-Rbx1 neddylation-Cullin-RING-Ligase proteins are essential for the maintenance of Regulatory T cell fitness

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Neddylation-mediated activation of Cullin-RING E3 Ligases (CRLs) are necessary for the degradation of specific immune regulatory proteins. However, little is known about how these processes govern the function of regulatory T (Treg) cells. Here we show that mice with Treg cell-specific deletion of Rbx1, a dual E3 for both neddylation and ubiquitylation by CRLs, develop an early-onset fatal inflammatory disorder, characterized by disrupted Treg cell homeostasis and suppressive functions. Specifically, Rbx1 is essential for the maintenance of an effector Treg cell subpopulation, and regulates several inflammatory pathways. Similar but less severe phenotypes are observed in mice having Ube2m, a neddylation E2 conjugation enzyme, deleted in their Treg cells. Interestingly, Treg-specific deletion of Rbx2/Sag or Ube2f, components of a similar but distinct neddylation-CRL complex, yields no obvious phenotype. Thus, our work demonstrates that the Ube2m-Rbx1 axis is specifically required for intrinsic regulatory processes in Treg cells; and that Rbx1 might also play Ube2m-independent roles in maintaining the fitness of Treg cells, suggesting a layer of complexity in neddylation-dependent activation of CRLs.
CRLs are a family of multi-unit E3 ubiquitin ligases complex, consisting of 4 subunits: a scaffold cullin with 8 family members, an adaptor with many members, a substrate receptor with hundreds of members, and a RING component with two family members, RING-box 1 (Rbx1/Roc1) and RING-box 2 (Rbx2/Roc2/Sag)\(^1\). The two members of RING family act as the catalytic subunit: Rbx1 couples mainly with CRL52. By both murine and human allogeneic T cell responses\(^22\), release of proinflammatory cytokines\(^15\) and CRLs regulate immune system, even though both neddylation/CRLs and Treg cells play a fundamental role in immune modulation.

In this study, we investigate whether and how Rbx1-Rbx2/CRLs and Ube2m-Ube2f/neddylation regulate the homeostasis and function of Treg cells, and find that while mice with a Treg-specific deletion of Rbx2/Sag or Ube2f show no obvious phenotypes, mice with Rbx1 deletion in Treg cells develop an early-onset fatal inflammatory disorder and death at day ~25 after birth (~p25), with impaired suppressive functions and disrupted homeostasis of Treg cells, indicating Rbx1 as a prominent regulator of Treg cells. Moreover, mice with Ube2m deletion in Treg cells also suffers from inflammatory disorders, but to a much lesser severity with a 50% of death rate at ~150 days of age. The immune suppressive function of Treg cells deficient of either Rbx1 or Ube2m are severely or moderately compromised, respectively. Unbiased transcriptome comparison between Rbx1-deficient and Ube2m-deficient Treg cells reveals an overlapping but also unique changes in the signaling pathways controlling the inflammatory responses with the former showing greater alterations. Our study suggests that targeting CRL-neddylation could be an effective approach for the treatment of human diseases with over-activated Treg cells.

Results

Early-onset fatal inflammation in Foxp3\(^{cre}\)Rbx1\(^{fl/fl}\) mice

To investigate the role of CRLs in the regulation of Treg cells in vivo, we generated conditional knockout mouse models with inactivation of Rbx1 and Rbx2/Sag individually in Treg cells, driven by Foxp3\(^{YP-cre}\) (Foxp3\(^{cre}\))\(^22,28\). The compound mice were designated as Foxp3\(^{cre}\)Rbx1\(^{fl/fl}\) or Foxp3\(^{cre}\)Sag\(^{fl/fl}\) mice, respectively. Although Sag was expressed in \(T\) cells and mice with loss-of-function deletion of Sag (Supplementary Fig. 1a) does not obviously impair Treg cell function and survival at the steady state (Supplementary Fig. 1b–d). Specifically, compared to Foxp3\(^{cre}\) controls (wild-type/\(T\) cells), Foxp3\(^{cre}\)Sag\(^{fl/fl}\) mice were viable and healthy without obvious alterations in activation markers of CD4\(^+\)Foxp3\(^-\) T cells (conventional T cells, or Tcon cells) (Supplementary Fig. 1c), the Tcon cell proliferation rate (Supplementary Fig. 1c), and the Treg/CD4\(^+\) ratio (Supplementary Fig. 1d), suggesting that the role of Sag in Treg cells at the steady state is, if any, not obvious.

Strikingly, deletion of Rbx1 in Treg cells (Supplementary Fig. 2a) was early-onset fatal. Compared to \(w\) controls, Foxp3\(^{cre}\)Rbx1\(^{fl/fl}\) mice showed an altered appearance as early as day 13–15 after birth (p13–15), with collapsed ears, festered skin, and reduced body size (Fig. 1a). The mice continued to lose weight dramatically after p15 (Fig. 1b) with a 50% or 100% of death rate at p25 or p37, respectively (Fig. 1c). Autopsy examination revealed that Foxp3\(^{cre}\)Rbx1\(^{fl/fl}\) mice had swollen lymph nodes and spleens (Fig. 1d, e), with lymphocyte infiltration into multiple organs, including the skin, lung, stomach, liver, kidney, and colon (Supplementary Fig. 2b). The Foxp3\(^{cre}\)Rbx1\(^{fl/fl}\) mice also had a decreased CD4\(^+\)/CD8\(^+\) T-cell ratio (Supplementary Fig. 3a, b) and a significantly increased proportion of effector/memory T cells (CD4\(^+\)/CD62L\(^{lo}\)) among populations of Tcon cells (Fig. 1f and Supplementary Fig. 3c). Beyond CD4\(^+\)- T cells, multiple types of immune cells, including CD8\(^+\) cytotoxic T cells, macrophages, dendritic cells, B lymphocytes, were all dramatically activated in Foxp3\(^{cre}\)Rbx1\(^{fl/fl}\) mice (Supplementary Fig. 4), indicating a typical phenotype of autoimmune disease. Such robust inflammation observed in Foxp3\(^{cre}\)Rbx1\(^{fl/fl}\) mice is reminiscent of Treg-deficient mice\(^23\) or mice with loss-of-function mutations in the Foxp3 gene\(^29\), suggesting Rbx1 is absolutely essential for Treg cells in vivo.
Fig. 1 Rbx1 deletion in Treg cells leads to an early-onset fatal inflammatory disorder. a Representative images of wt and Foxp3<sup>fl/fl</sup>;Rbx1<sup>1/1</sup> mice (p20, male, scale bar = 1 cm). b Gross body weight of wt and Foxp3<sup>fl/fl</sup>;Rbx1<sup>1/1</sup> mice (p19–23, n = 10 animals, for gender information see "source data"). c Survival curve of wt and Foxp3<sup>fl/fl</sup>;Rbx1<sup>1/1</sup> mice (n = 21 animals from both male and female mice, p < 0.0001). d Representative images of the peripheral lymph nodes (top) and spleen (bottom) from wt and Foxp3<sup>fl/fl</sup>;Rbx1<sup>1/1</sup> mice (p22, male, scale bar = 1 cm). e Total cell numbers in peripheral lymph nodes and spleen from wt and Foxp3<sup>fl/fl</sup>;Rbx1<sup>1/1</sup> mice (p19–23, n = 10 biologically independent samples from both male and female mice, p < 0.0001 for left, and p = 0.2445 for right). f Expression of CD44 and CD62L in Tcon cells from peripheral lymph nodes of wt and Foxp3<sup>fl/fl</sup>;Rbx1<sup>1/1</sup> mice (p21, male). g The levels of Il-17A and Il-4 in Tcon cells from peripheral lymph nodes of wt and Foxp3<sup>fl/fl</sup>;Rbx1<sup>1/1</sup> mice (p19–23, n = 5 biologically independent samples from both male and female mice, p < 0.0001 for left, and p = 0.0011 for right). h The levels of Il-17A in Tcon cells from peripheral lymph nodes of wt and Foxp3<sup>fl/fl</sup>;Rbx1<sup>1/1</sup> mice (p19–23, n = 5 biologically independent samples from both male and female mice, p = 0.0046). All error bars represent the SEM, data are presented as mean values ± SEM. The p values were calculated by Mann-Whitney test. Source data are provided as a Source Data file.

The levels of Il-17 and Il-4 were remarkably increased in Tcon cells by flow cytometry detection of single cell suspension from lymph-nodes (Fig. 1g and Supplementary Fig. 3d). In addition, the serum concentrations of Th1/Th12 cytokines (Il-17, Il-2, Th1, Th2, Il-6, Th1, Th10, and Th13) for Th1/Th12 (Supplementary Fig. 5a, b) and antibodies (IgA and IgG2a for Th1; IgE and IgG1 for Th2) (Supplementary Fig. 6a, b) were all elevated and, indicating a robust activation of Th1/Th12-immune responses. For Th17 reaction, flow cytometry of single cell suspension from lymph-nodes revealed a two-fold and statistically significant increase of Il-17A in Tcon cells (Fig. 1h and Supplementary Fig. 3e), and serum concentrations of Th17 antibodies (IgG3, IgG2b) were also elevated, although not reaching the statistical difference (Supplementary Fig. 6c). While the serum concentrations of Th17 cytokines (Tnf-α, and GM-CSF) did not change obviously, the Il-17A level was actually reduced (Supplementary Fig. 5c), despite a robust inflammatory storm was evident with significantly increased amount of various cytokines in Foxp3<sup>fl/fl</sup>;Rbx1<sup>1/1</sup> mice (Supplementary Fig. 5d). Thus, while the Th17 reaction was activated modestly, an excessive Th1/Th2-dominant immune polarization was clearly shown in Foxp3<sup>fl/fl</sup>;Rbx1<sup>1/1</sup> mice.

Rbx1 is required for the immune suppressive function of Treg cells. To investigate whether the suppressive function of Rbx1-deficient Treg cells was impaired, we performed an in vivo suppression assay. The equal number of Treg cells from wt or Rbx1-deficient mice were injected into immune-deficient Rag1<sup>−/−</sup> mice, along with naive conventional CD4<sup>+</sup> T cells. If mice develop the colitis in 4 weeks, induced by co-injected naive conventional CD4<sup>+</sup> T cells, it would indicate that Treg cells from Rbx1-deficient mice have lost suppression function, and vice versa. We found that while wt Treg cells prevent the colitis, as evidenced by a normal colon morphology, the Rbx1-deficient
Treg cells failed to do so, and the mice developed very severe colitis, similar to the control mice without receiving Treg cell injection (Fig. 2a), demonstrating that the immune suppressive function of Treg cells was indeed impaired upon \textit{Rbx1}-deletion.

To exclude the possibility that \textit{Rbx1}-deficient Treg cells might die in the 4-week period during the in vivo suppression assay, we also used an alternative strategy to investigate the impairment of the immune suppressive function of \textit{Rbx1}-deficient Treg cells at early age. Compared to the \textit{wt} control mice, \textit{Foxp3\textsuperscript{cre};Rbx1\textsuperscript{fl/fl}} mice at the p8 stage have similar levels of Treg/CD4\textsuperscript{+} ratio (Fig. 2b), similar numbers of Treg cells, as well as total numbers of lymphocytes (Fig. 2c, d). However, these Treg \textit{Rbx1}-deficient
infant mice still showed an over-activated immune system with a ~3–4-fold increase in proportion and cell number of effector/memory (CD44hiCD62Llo) T cells (Fig. 2e).

Finally, we performed the wt Treg infusion experiment and found that intraperitoneal injection of wt Treg cells to the infant Foxp3cre;Rbx1fl/fl mice cannot rescue the fatal inflammation (Fig. 2f, g). Specifically, compared to wt control, Foxp3cre;Rbx1fl/fl mice (at the p8 stage) transplanted with wt Treg cells still developed immune over-activation (harvested at the p20 stage), revealed by elevated proportion of effector/memory T cells (CD44hiCD62Llo) among populations of Tcon cells (Fig. 2f), and increased proliferation rate of Tcon cells (Fig. 2g). The possible explanation is that despite Treg cells were reported to inhibit established inflammation in 5 weeks, the infused wt Treg cells did not have enough time window for functional rescue after inflammation had already started in the recipient infant Foxp3cre;Rbx1fl/fl mice. Taken together, all these three assays demonstrated that Rbx1 is essential and required for immune suppression function of Treg cells.

In moribund Foxp3cre;Rbx1fl/fl mice at p19–23 stage, Treg cellular compartment was reduced dramatically, as evidenced by reduced Treg cell ratios (Supplementary Fig. 7a) and actual numbers (Supplementary Fig. 7b), with reduced proliferation and increased apoptosis, revealed by reduced Ki67 expression (Supplementary Fig. 7c) and increased Caspase-3 activity (Supplementary Fig. 7d). Thus, the fatal inflammatory disorders in Foxp3cre;Rbx1fl/fl mice are attributable to both impaired suppressive function and decreased cellular compartment.

**Rbx1 is essential for the conversion of quiescent to effector subpopulations of Treg cells.** Single-cell RNA sequencing (scRNA-seq) was utilized to unbiasedly dissect the cellular heterogeneity of wt vs. Rbx1-deficient CD4 T cells from the peripheral lymph nodes of inflammation-free female Foxp3cre/wt and Foxp3cre/wt;Rbx1fl/fl mice (Note that the Foxp3 gene is localized in the X-chromosome, and the female mice used here with two genotypes are chimeric, Supplementary Fig. 8). As revealed in the two-dimensional Uniform Manifold Approximation and Projection (UMAP) of the single-cell transcriptomics data, both wt and Rbx1-deficient Treg cells could be divided into several subpopulations (Fig. 3a and Supplementary Fig. 9). Rbx1 depletion increased the subpopulation in Cluster 0, but remarkably decreased the subpopulations on Clusters 3 and 4 without obviously affecting other subpopulations (Fig. 3a, b).

The top 10 genes and top 10 pathways in each of 14 clusters in Treg cells were shown (Supplementary Figs. 10, 12). Among them, the cells in Cluster 0 expressed high levels of genes characteristic of naive Treg cells, including *Sell* (Cd62L) and *Bcl2*; while Treg cell functional molecules, such as *Cita* (C34), *Icos* (I34), and *Cd44* (I35), were highly expressed in Cluster 3 and 4 (Fig. 3c). To confirm this finding, we performed the FACS-based profiling and found a ~3–4-fold reduction of CD44 and CD62L in Treg cells from peripheral lymph nodes in Foxp3cre;Rbx1fl/fl mice at age of ~20-day (Fig. 3d and Supplementary Fig. 11a). A similar reduction was also observed in infant Foxp3cre;Rbx1fl/fl mice at age of 8-day (Fig. 3e and Supplementary Fig. 11b), indicating that the reduction in Cluster 3/4 subpopulations upon Treg Rbx1-deletion is an early event.

The KEGG pathway enrichment analysis revealed that, compared to Cluster 0, T-cell receptor and other Treg-related pathways were activated in Clusters 3 and 4 (Fig. 3f and Supplementary Fig. 12). Thus, Cluster 0 is a quiescent subpopulation, designated as the center population, whereas Clusters 3 and 4 are active Treg cell subpopulations, designated as effector populations. The fact that the loss of Rbx1 leads to the failure in the conversion of quiescent subpopulation to effector subpopulation supports the notion that Rbx1 is essential for such a conversion in the Treg cells.

**Disordered functional and regulatory network in Rbx1-deficient Treg cells.** Transcriptome analysis of Rbx1-deficient Treg cells from inflammation-free female Foxp3cre/wt;Rbx1fl/fl mice (Supplementary Fig. 8) revealed significant down-regulation of functional genes, including *Il10*, *Tgfβ*, and *Csf* (suppressing cytokines); *Il2*, *Entpd1*, and *Nrp1* (regulators of immune cell metabolism); *Cita*, *Icos*, *Nrp*, and *Lag3* (inhibitor via dendritic cells); and *Fas* and *Lgals1* (apoptosis inducer). Impairment of migration-associated surface molecules was also observed in Rbx1-deficient Treg cells, including downregulation of *Cd44*, *Cxcr3*, *Cxcr5*, and *Cxcrl* and upregulation of *Sell* (Fig. 4a).

The KEGG pathway enrichment analysis revealed that Rbx1 depletion caused downregulation of transcriptome involved in many signaling pathways, including T-cell receptor, Th17 cell differentiation, chemokine and cytokine–cytokine receptor interaction, NOD-like receptor, TNF, Toll-like receptor, Hippo, TGFB, NF-kB, PI3K-AKT, and purine metabolism among few other virus associated pathways (Fig. 4b and Supplementary Fig. 13). The results suggest that loss of Rbx1 results in changes in the expression of numerous key genes regulating the processes of inflammation, immunological responsiveness, proliferation/survival, and metabolisms, which are subjected to further experimental validation.

Considering Rbx1 is an E3 ubiquitin ligase that directly controls the abundance of substrate proteins, we utilized the mass spectrometry-based proteomics to profile the difference in protein levels between wt and Rbx1-deficient CD4 T cells (three independent sets of combined samples) from inflammation-free female Foxp3cre/wt and Foxp3cre/wt;Rbx1fl/fl mice (Supplementary Fig. 8). Among a total of 3236 proteins detected in any one of 6 paired samples, we identified 43 increased and 78 decreased proteins with a three-fold cut-off, and the numbers increased to 70 and 195, respectively, with a two-fold cut-off in Rbx1-deficient CD4 T cells, as compared to the wt control. The KEGG pathway enrichment analysis of...
Fig. 3 Single-cell transcriptomics of wt and Rbx1-deficient Treg cells. a Two-dimensional UMAP visualization of single cell transcriptomics data in CD4\(^+\)YFP\(^+\) Treg cells from peripheral lymph nodes of female Foxp3\(^{cre}\)/wt and Foxp3\(^{cre}\)/wt;Rbx1\(^{-/-}\) mice (10 weeks old). b Proportions of each cluster in CD4\(^+\)YFP\(^+\) Treg cells in peripheral lymph nodes from female Foxp3\(^{cre}\)/wt and Foxp3\(^{cre}\)/wt;Rbx1\(^{-/-}\) mice (10 weeks old). c Heat map of selected marker genes in clusters 0, 3, and 4. Single cells were represented by vertical lines and different colors reflected the relative abundance of indicated genes. d Expression of CD44 and CD62L in Treg cells from peripheral lymph nodes of wt and Foxp3\(^{cre}\)/wt mice (p20, male). e Expression of CD44 and CD62L in Treg cells from peripheral lymph nodes of infant wt and infant Foxp3\(^{cre}\)/Rbx1\(^{-/-}\) mice (p8, male). f Selected pathways of clusters 0, 3, and 4 in the KEGG analysis. Source data are provided as a Source Data file.
two-fold cut-off revealed that Rbx1 depletion caused an obvious change of many pathways (Supplementary Fig. 14), including accumulation of few proteins associated with the splicesome or complement and coagulation cascade, but significant reduction of proteins involving ER processing, and of proteins controlling the various metabolic pathways, including TCA cycle, carbon and purine metabolisms, along with other metabolic pathways (Fig. 4c, d). We marked (with arrows) the genes with the consistent changes at both mRNA and protein levels, and these observed changes at the protein levels are likely due to altered gene transcription (Fig. 4c, d), which is again subjected to further experimental validation.

Specific spectrum of Rbx1 substrates in Treg cells. To define candidates as possible direct substrates of Rbx1 ubiquitin E3 ligase in Treg cells, the proteomic and transcriptomic data were compared directly. We reasoned that the substrate candidates should be those with the increase at the levels of protein, but not of mRNA, in Rbx1-deficient Treg cells. A total of 57 such candidates were identified (Fig. 5a). While Aty17, Bcl2l11/Bim46, Ets149, Nkhh1/biXb–p2740, and Zhex51 were previously reported as Rbx1 substrates, suggesting the reliability of our methodology, several others in the list are likely the novel substrates for future validation.

Interestingly, many common substrates of Rbx1/CRLs1-41,46 were not detected in Rbx1 deficient Treg cells, and few detected ones, like Cdkn1b/p2747 and Foxo152 were not accumulated (Fig. 5b). Thus, it appears that only a small subset of Rbx1 substrates are selectively expressed, and specifically subjected to Rbx1 modulation in Treg cells to maintain their proper functions.

The involvement of Bim in the phenotypes of Foxp3cre;Rbx1fl/fl mice. In order to validate these putative Rbx1 substrates in Treg cells, we searched all 57 proteins for antibodies commercially available for FACS-based quantification, given the difficulty in harvesting sufficient Treg cells for traditional Western blotting analysis. Unfortunately, only available antibody is for Bim. We, therefore, performed the FACS analysis, as described52, to quantify the levels of Bim, and confirmed that Bim is indeed accumulated up to three-fold in Rbx1-depleted Treg cells (Fig. 6a, b). It is noting that our single cell RNA sequencing data revealed that Bim mRNA is widely expressed in all subpopulations of Treg cells (Supplementary Fig. 15a).

Given Bim is a pro-apoptotic protein54, and also involved in Treg cells regulation55, Bim accumulation could mediate at least in part some phenotypes of Rbx1-deficient Treg cells. We tested this working hypothesis via a Bim rescue experiment by generating and characterizing conditional knockout mice with inactivation of both Rbx1 and Bim in Treg cells driven by Foxp33FP-cre (Foxp3cre46,27,28 (designated as Foxp3cre;Rbx1fl/fl;Bimfl/fl mice), which have more than 10-fold reduction of Bim levels in Treg cells (Supplementary Fig. 15b). The Foxp3cre;Rbx1fl/fl;Bimfl/fl mice also developed an early-onset fetal inflammatory disorder with very short lifespan, similar to the Foxp3cre;Rbx1fl/fl mice (Fig. 6c, d), along with decreased CD4+CD8+ T-cell ratio (Supplementary Fig. 15c) and significantly increased proportion of effecter/memory T cells (CD44hiCD62Llo) among populations of Tcon cells (Fig. 6e and Supplementary Fig. 15d). Notably, the above inflammation markers, however, were milder in double cKO Foxp3cre;Rbx1fl/fl;Bimfl/fl mice than those in Foxp3cre;Rbx1fl/fl mice, with the statistically significant p values (Fig. 6e, f), suggesting the inflammation and immune over-activation induced by Rbx1-deficiency in Treg cells were moderately attenuated by Bim-deletion. Moreover, Treg ratio among CD4+T cells in Foxp3cre;Rbx1fl/fl;Bimfl/fl mice remained significantly reduced, as compared to wt control (Fig. 6g and Supplementary Fig. 15e), despite slightly higher than that in Foxp3cre;Rbx1fl/fl mice (Fig. 6h). However, the absolute numbers of Treg cells in Foxp3cre;Rbx1fl/fl;Bimfl/fl mice were comparable to the wt control mice (Supplementary Fig. 15f), while it was significantly reduced in Foxp3cre;Rbx1fl/fl mice (Supplementary Fig. 7b). Notably, Treg cells with double deletion of Rbx1 and Bim still suffer from elevated apoptosis (Supplementary Fig. 15g), in a level similar to Rbx1-deficient Treg cells (Supplementary Fig. 7d). Taken together, Bim deletion in Treg cells attenuated the severe inflammation phenotype, although it failed to rescue the survival of Foxp3cre;Rbx1fl/fl mice, indicating Bim is partially involved in the function of Rbx1 in Treg cells.

Later-onset fatal inflammation in Foxp3cre;Ube2mfl/fl mice with impaired suppressive function of Treg cells. It has been well-recognized that the E3 ligase activity of the CRLs requires neddylation of the scaffold protein Cullin56,57. Specifically, neddylation E2 Ube2m couples with Rbx1 (neddylation co-ES3) selectively promotes neddylation of Cullins 1–4, which are complexed with Rbx1 to constitute active CRLs 1–4 ubiquitin E3; whereas neddylation E2 Ube2f couples with Sag/Rbx2 E3 to promote neddylation of Cullin 5, which is then complex with Sag/Rbx2 to constitute active CRL52. We, therefore, asked whether the pivotal role of Rbx1 in Treg cells is dependent on Ube2m, whereas Ube2f is non-essential for Treg cells, given the non-essentiality of Sag/Rbx2 (Supplementary Fig. 1). Indeed, despite Ube2f is expressed in wt Treg cells (Supplementary Fig. 16a), Foxp3cre;Ube2f−/−mice with Ube2f deletion in Treg cells are viable and develop normally without any abnormality, as evidenced by a normal level of an activation marker of CD4+Foxp3+ T cells (Supplementary Fig. 16b), a normal rate of Tcon cell proliferation (Supplementary Fig. 16c), and a normal Treg/CD4+ ratio (Supplementary Fig. 16d), indicating that the Ube2f-Rbx2-CRL5 axis is not essential for the functionality and survival of Treg cells at the steady status.

On the other hand, like Foxp3cre;Rbx1fl/fl mice, Foxp3cre;Ube2mfl/fl mice with Ube2m deletion in Treg cells (Supplementary Fig. 17a), displayed abnormal appearances such as collapsed ears and festered skin, but at the much later stage (Fig. 7a). The growth of mice was remarkably retarded (Fig. 7b), and about 50% of the mice dies at age of 150 days and a 100% death rate within one year (Fig. 7c). The swollen spleens and lymph nodes were apparent with increased cellularity (Fig. 7d and Supplementary Fig. 17b) with moderate lymphocyte infiltration into multiple organs, including skin, lung, stomach, liver, kidney, and colon (Supplementary Fig. 17c). Furthermore, the Foxp3cre;Ube2mfl/fl mice also showed obvious T1h1/T1h2-polarized immune over-activation of multiple immune cells (Fig. 7e–g and Supplementary Figs. 18–21), but in a much lower degree of inflammation than that in Foxp3cre;Rbx1fl/fl mice (Fig. 1 and Supplementary Figs. 2–6).

Interestingly, unlike Foxp3cre;Rbx1fl/fl mice, Foxp3cre;Ube2mfl/fl mice had increased Treg/CD4+ :T ratio and increased number of Treg cells (Fig. 8a) in lymph nodes, without obvious alterations in the levels of Ki67 and active Caspase-3 in Treg cells (Fig. 8b, c). It has been shown that in response to inflammation insult, the immuno-suppressive Treg cells usually proliferate to inhibit the inflammation and restore the immune homeostasis of the bod58,59. The ratio (Treg/CD4+) and number of Treg cells are, therefore, elevated in vivo under inflammation, if the proliferation and survival of Treg cells are not inhibited, which is the case for Ube2m-deficient Treg cells, but not for Rbx1-deficient Treg cells. Nevertheless, the fact that Foxp3cre;Ube2mfl/fl mice still developed over-reactive immune response (Fig. 7) strongly suggested
the impairment of suppressive function of Ube2m-deficient Treg cells. Indeed, the in vivo suppression assay using immune-deficient Rag1−/− mice clearly showed that very severe colitis, induced by injection of naive CD4⁺ T cells, cannot be inhibited by Ube2m-deficient Treg cells (Fig. 8d). However, unlike that of Foxp3^{cre};Rbx1^{fl/fl} mice, the inflammatory immune phenotype of Foxp3^{cre};Ube2m^{fl/fl} mice can be largely rescued by intraperitoneal injection of wt Treg cells, as evidenced by a largely normal proportion of both effector/memory T cells (CD44hiCD62Llo) among populations of Tcon cells (Fig. 8e) and proliferation rate of Tcon cells (Fig. 8f), suggesting that impaired immune suppressive function in Foxp3^{cre};Ube2m^{fl/fl} mice are relatively
minor, as compared to Foxp3cre;Rbx1fl/fl mice and can be rescued by wt Treg cells in this infusion experiment.

Comparison of signaling pathways regulated by Rbx1 and Ube2m in Treg cells. The phenotypes between Foxp3cre;Rbx1fl/fl and Foxp3cre;Ube2mfl/fl mice clearly demonstrate the similarities and differences between Rbx1 and Ube2m in the maintenance of the functionality of Treg cells. To dissect them at the molecular levels, we performed the genome-wide transcriptome analyses of CD4+ YFP+ Treg cells derived from female Foxp3cre/wt and Foxp3cre/wt;Rbx1fl/fl mice (8-10 weeks old). KEGG analysis was performed and genes with changes greater than 2 times and p value less than 0.05 (p < 0.05) were selected; biologically significant and interesting pathways were selected and displayed in the heat-map. c, d Comparison of the levels of proteins vs. mRNAs in genes associated with indicated pathways in CD4+ YFP+ Treg cells from female Foxp3cre/wt and Foxp3cre/wt;Rbx1fl/fl mice (8-10 weeks old). The mRNAs with greater than 1.3-fold changes, which are consistent with protein level changes, were marked by the arrows. KEGG analysis was performed and proteins with changes greater than two times and p value less than 0.05 (p < 0.05) were selected; biologically significant and interesting pathways were selected and displayed in the heat-map.

Discussion

In this study, we systematically investigated the role of CRLs and neddylation in the maintenance of fitness of Treg cells, using four Treg selective KO mouse models. While the Ube2f/Sag axis plays a minimal, if any, role, the Ube2m/Rbx1 axis is indeed essential for the homeostasis and survival of Treg cells, although all four genes are widely expressed in multiple mouse tissues and in all subpopulations in Treg cells (Supplementary Fig. 26). The results showed the similar changes in the Treg functional genes (Supplementary Fig. 22), and multiple signaling pathways involved in inflammation responses, but generally to a lesser extent in Ube2m-deficient Treg cells (Supplementary Fig. 23). An unbiased cluster analysis of the transcriptomes between Rbx1-deficient and Ube2m-deficient Treg cells revealed a good overall correction, but two sets of data did have unique differences in several groups of genes and pathways, particularly seen in Rbx1-deficient Treg cells (Fig. 9a and Supplementary Figs. 24, 25), demonstrating that CRLs and neddylation did have common as well as unique mechanisms in the regulation of Treg cell functions.

Taken together, both the phenotypic comparison and unbiased cluster analyses of the transcriptome support the notion that Rbx1 plays Ube2m-dependent and independent roles in Treg cells (Fig. 9b).

Several lines of evidence suggest that severe autoimmune/inflammatory phenotype of Foxp3cre;Rbx1fl/fl mice is not the simple consequence of reduced proliferation and/or enhanced apoptosis, rather it is also attributable to the impaired immune suppression of Rbx1-deficient Treg cells. First, in an in vivo suppression assay, Rbx1-deficient Treg cells failed to inhibit the colitis induced by naive CD4+ T cells in immune-deficient Rag1−/− mice (Fig. 2a); Second, the 8-day-old infant Foxp3cre;Rbx1fl/fl mice with normal ratio of Treg/CD4⁺-T cells and normal number of Treg cells already showed the immune activation (Fig. 2b-e); third, infusion of wt Treg cells into Foxp3cre;Rbx1fl/fl mice failed to rescue inflammation phenotype within the testing time window (Fig. 2f, g); and forth, simultaneous deletion of Bim and Rbx1 failed to extend mouse life-span and rescued the severe inflammation phenotypes (Fig. 6).

So far, the only reported study of CRL regulation of Treg cells was the Treg deletion of Vhl, a substrate receptor of CRL2 for targeted degradation of Hif1α. In that study, the authors showed that mice with Vhl-deficient Treg cells also developed severe inflammatory disorders with a 50% of death rate at the age of 8 weeks. Mechanistically, the production of Vhl-deficient Treg cells of a large amount of Ifn-γ due to transactivation by accumulated Hif1α is responsible for immune-disorder phenotype, which was completely rescued by simultaneous Ifn-γ deficiency in Treg cells. In contrast, the production of Ifn-γ by Rbx1-deficient Treg cells is comparable with that of wt controls (Supplementary Fig. 27), although other T cells (non-Treg cells) produced more Ifn-γ (Fig. 1g and Supplementary Fig. 3d), indicating a different mechanism of action between Vhl and Rbx1 in functional regulation of Treg cells.

Our Microwell-based scRNA-seq revealed unbiasedly that Treg cells were divided into several subpopulations, and the quiescent population was significantly increased, whereas the effector subpopulations were significantly reduced upon Rbx1 depletion (Fig. 3), highly suggesting a failure in the conversion of the quiescent subpopulations into effector ones, rather than the death of these effector cells. Mechanistically, although we identified several altered signaling pathways (Fig. 3f), how exactly Rbx1 depletion failed to trigger such a conversion is currently unknown. Given the major role of Rbx1 as a dual E3 in both neddylation and ubiquitylation, it is very likely that Rbx1 depletion would result in the abnormal levels or activities of transcription factors and/or signaling molecules directly (via substrate accumulation or neddylation modification) or indirectly (altered transcriptions due to direct effect). Future studies are geared to elucidate the underlying mechanism. On the other hand, the fact that Rbx1-deficiency has no effect on other subpopulations of Treg cells provides an ideal opportunity of Rbx1 targeting for precise manipulation of Treg cell subpopulations.

It is worth noting that droplet-based and plate-based strategies were previously used for the scRNA-seq transcriptomics analysis
provided informative knowledge as to how Rbx1 regulates Treg subpopulations. Furthermore, this study is the first to provide detailed informative heat-map of top 10 characteristic genes in each of 14 clusters via scRNA sequencing, along with associated pathways (Supplementary Figs. 10, 12).

Both proteomics and transcriptome approaches were employed to analyze the effect of Rbx1-deficiency on Treg cells at the molecular levels. We noticed that less than half of protein changes seen in the proteomic analysis matched with the mRNA changes by the transcriptomic analysis (Fig. 4c, d). While this is slightly lower than the general correlation of 50% between proteome and transcriptome\(^6\), the correlation between proteome and transcriptome is known to be distinct in different tissues or cell types\(^6\), including in human Treg cells, as reported recently\(^6\), likely due to the low abundance of Treg population in vivo with limited detection in our study of only ~3200 proteins in any given 6 samples by Mass Spectrum analysis. The comparison of proteome and transcriptome of Treg cells should provide some cues for future investigation of detailed mechanisms of Treg cell regulation by CRLs and neddylation.

The combination of proteomic and transcriptomic data revealed several Treg-specific Rbx1 degradation candidates, including Acly\(^47\), Bcl2l11/Bim\(^48\), Ets1\(^49\), Nkfbib/IkBa\(^50\), and Zhx2\(^51\). Among the putative substrates of Rbx1 in Treg cells, Nkfbib/IkBa is the inhibitor of NF-kB pathway, Fto is the demethylation enzyme of m6A mRNA methylation, Ets1 is a transcription repressor, whereas Bcl2l11/Bim is a pro-apoptotic protein. The NF-kB pathway\(^28,67\), m6A mRNA methylation\(^68\), and Bcl2l11/Bim\(^55\) are all known to regulate Treg cell. Our Bim rescue experiment showed a partially role of Bim in Treg Rbx1-deficient phenotypes (Fig. 6), suggesting the severe inflammation phenotype is likely caused by the combination of multiple substrates. Future studies should be directed to characterize the substrates, particularly those involved in the regulation of global gene expressions (such as Fto and Ets1) in the functional regulation of Treg cells upon Rbx1 deletion.

While Treg Ube2m-deficiency also causes obvious inflammation disorders (Fig. 7), the phenotype severity is much lesser than that seen in Treg Rbx1-deficient mice (Fig. 1). Mechanistically, although both Rbx1 and Ube2m controls the expression of genes regulating several inflammation-related pathways (Supplementary Fig. 23), the downregulation of Egr2\(^29\), Ccr7\(^70\) and Tigit\(^71\) is rather unique to Rbx1-deficient Treg cells (Fig. 9a and Supplementary Fig. 24). Altered expression of these genes, which has been previously shown to regulate Treg cell function, would likely be responsible for the more severe phenotype seen in Foxp3\(^{3cre}Bx1^{flfl}\) mice.

In summary, we used in this study three comprehensive approaches, namely single cell sequencing, whole transcriptome profiling, and mass-spect based proteomics, in an attempt to define the mechanism of Rbx1 action in Treg cells. Given the broad role of the neddylation-CRLs in fundamental biological processes, our study yielded a wealth of information on many altered signaling pathways and individual gene/protein upon Rbx1 Treg deletion. We reason that this is the first overall
phenotypical study of elucidating the role of neddylation enzymes, particularly Rbx1/Ube2m, in Treg cells, which sets the foundation for future detailed studies to pinpoint special role of each interesting pathways and individual genes. Given that inactivation of either neddylation E2/Ube2m, or E3/Rbx1 caused suppression of Treg function, MLN4924, a small molecule inhibitor of neddylation, currently in several Phase II clinical trials for anticancer application3,6 may have a new application in the treatment of diseases associated with over-activation of Treg cells, which is certainly worth investigating experimentally.

Fig. 6 Involvement of Bim in the phenotypes of Foxp3cre;Rbx1fl/fl mice. a Bim in Treg cells from peripheral lymph nodes of wt and Foxp3cre;Rbx1fl/fl mice (p19, male). b The proportion of Bimhi cells among Treg cells from peripheral lymph nodes from wt and Foxp3cre;Rbx1fl/fl mice (p19–21, n = 3 biologically independent samples from both male and female mice). c Representative images of wt and Foxp3cre;Rbx1fl/fl mice (p19–21, n = 3 biologically independent samples from both male and female mice). d Survival curve of wt, Foxp3cre;Rbx1fl/fl and Foxp3cre;Rbx1fl/fl;Bimfl/fl mice (n = 16 animals for Foxp3cre;Rbx1fl/fl;Bimfl/fl mice from both male and female mice). e Expression of CD44 and CD62L in Tcon cells from peripheral lymph nodes of wt and Foxp3cre;Rbx1fl/fl;Bimfl/fl mice (p20, male). f CD44hi/CD62Llo/Tcon ratios in peripheral lymph nodes from wt, Foxp3cre;Rbx1fl/fl and Foxp3cre;Rbx1fl/fl;Bimfl/fl mice (p19–23, n = 11, 4, or 7 biologically independent samples respectively from both male and female mice). All error bars represent the SEM, data are presented as mean values +/− SEM. The p values were calculated by Mann–Whitney test. Source data are provided as a Source Data file.
**Methods**

**Mice.** The Foxp3cre;Ube2mfl/fl mice (Jax No. 016959) are gifts from Prof. Xiaoming Feng (Peking Union Medical College, China).

The B6x129 strain mice were generated on the C57BL/6 background via CRISPR/Cas9 technology with exons 2–4 floxed, performed by GemPharmatech Co., Ltd. (Nanjing, China). Briefly, the sgRNA (5'-ACCTACAGTGATTGCTAC-3' and 5'-GAGTCAGAAATGATTGGTC-3') direct Cas9 endonuclease cleavage in intron1–2 and intron 4–5 to insert LoxP sites by homologous recombination. The PCR genotyping primers 5'-GGAGTCAGAAATGATTGGTC-3' and 5'-GGAGTCAGAAATGATTGGTC-3' direct Cas9 endonuclease cleavage in intron1–2 and intron 4–5 to insert LoxP sites by homologous recombination. The PCR genotyping primers 5'-AAGGTAGCAACATCTCAGTC-3' and 5'-TCTGATAGCAGGACGCAGGTC-3' generated DNA fragment of 266 and 196 bp bands, respectively, for mutant and wild-type.

The Ube2mfl/fl mice were generated on the C57BL/6 background, performed by GemPharmatech Co., Ltd. (Nanjing, China). The CRISPR/Cas9 technology was used to flox exons 2–4 with exon 5 frame-shifted in Ube2m genome. Given exon 1 only encodes 36 amino acids, there is no possibility that a truncated functional protein is still being made. Brie

The Bmxfl/fl mice were purchased from GemPharmatech Co., Ltd. (Nanjing, China) (T007185).

All mice were maintained in specific pathogen-free (SPF) conditions with experimental/control groups co-housed, and experiments were conducted...
with gender-matched experimental/control mice. Mice were euthanized via cervical dislocation or carbon dioxide. Permission was granted to perform animal experiments by the Animal Ethics Committee of Zhejiang University; animal care was provided in accordance with the principles and procedures by the regulatory standards at Zhejiang University Laboratory Animal Center. To request the mouse strains generated in this project, please contact Dr. Yi Sun, e-mail: yisun@zju.edu.cn.

Flow cytometry. Peripheral lymph nodes and/or spleens were harvested from mice with indicated genotypes, and grinded into single cells, subjected to followed experiments. For analysis of surface markers, cells were washed in PBS containing 2% (wt/vol) FBS, and stained with indicated Abs (dilution 1: 200); intracellular markers were strained with Transcription Factor Buffer Set (BD Pharmingen, 562574) according to the manufacturer’s instructions by indicated Abs (dilution 1: 50). For intracellular cytokine staining, cells were stimulated for 5 h with leukocyte...
activation cocktail of GolgiPlug (BD Pharmingen, 550583). Antibodies were all from eBioscience, unless otherwise noted: anti-CD4 (GK1.5), anti-CD8a (53-6.7, BD Pharmingen), anti-CD44 (IM7, BD Pharmingen), anti-CD62L (MEL-14), anti-Foxp3 (FJK-16s), anti-Ki67 (SolA15), anti-CD45 (30-F11), anti-F4/80 (BM8), anti-CD11b (MI70), anti-CD11c (N418), anti-MHCII (M11/15.2.5), anti-CD80 (16-10A1), anti-CD86 (GL1, BD Pharmingen), anti-CD220 (RA3-6B2), anti-CD19 (1D3, BD Pharmingen), anti-GL7 (GL7, BD Pharmingen), anti-Fas (15A7), anti-active caspase 3 (C92-605, BD Pharmingen), anti-Ifn-γ (XM1G.12), anti-Il4 (11B11), anti-Ili7A (Ebiol7B7), and anti-Bim (C343, CS Cell Signal Technology). Flow cytometry data were acquired on CytoFLEX LX (Beckman) and analyzed using Flowjo software (Tree Star).

**Cytokines assay.** The serum was drawn from mice of indicated genotypes, diluted four-fold by PBS, and then subjected to cytokine/chemokine profiling using Mouse Cytokine Grp I (Bio-Plex Pro, M60009RDPD), measured on Luminex 200 system.

**Antibodies assay.** The immunoglobulin subclasses in mouse serum were measured using Mouse Immunoglobulin Isotyping Kit (BD Cytometric Bead Array, 550026).

**Cell sorting.** Peripheral lymph nodes and/or spleens were harvested from mice with indicated genotypes, and grinded into single cells, followed by treatment with Dynabeads™ Untouched™ Mouse CD4 Cells Kit (Invitrogen, 11415D) to isolate the CD4+ T cells. The FACS sorting (SONY Cell Sorter, SH800S) was used to isolate the CD4+YFP+ Treg cells with purity >99%.

**The in vivo immune-suppression assay in Rag1−/− mice.** Naive T cells sorted by FACS (CD4+ YFP+ CD4−CD62L−, 4 × 10⁵) from Foxp3fl mice were transferred into Rag1−/− mice alone or in combination with indicated Treg cells (CD4+YFP−, 2 × 10⁵). Mice were euthanized at 4 weeks after T-cell transfer and the colon tissues were collected for histopathological analysis. The severity of colon damages were classified into five categories as described previously47: Grade 0 has normal colonic crypt architecture; Grade 1 has mild inflammation with slight epithelial cell hyperplasia and increased numbers of leukocytes in the mucosa; Grade 2 has moderate colitis with pronounced epithelial hyperplasia, significant leukocyte infiltration, and decreased numbers of goblet cells; Grade 3 has severe colitis with marked epithelial hyperplasia, extensive leukocyte infiltration, significant depletion of goblet cells, and occasional ulceration, or crypt abscesses; Grade 4 has very severe colitis with marked epithelial hyperplasia, extensive transmural leukocyte infiltration, severe depletion of goblet cells, severe ulceration, and many crypt abscesses.

**The in vivo adaptation of wt Treg cells.** The CD4+ YFP+ wt Treg cells were sorted from the Foxp3fl mice, and transplanted to the recipient by intraperitoneal injection. For Foxp3fl:Rbx1fl mice, 4 × 10⁵ wt Treg cells were transplanted to the Foxp3fl:Rbx1fl mice at p8 stage, and the recipients were euthanized at p20 for analysis. For Foxp3fl:Ube2mfl mice, 8 × 10⁵ wt Treg cells were transplanted to the Foxp3fl:Ube2mfl mice at 6 weeks old, and the recipients were euthanized at 16-week-old for analysis.

**Single-cell RNA sequencing.** CD4+ YFP+ Treg cells were isolated from the peripheral lymph nodes of mice with indicated genotypes as above. To generate enough materials, the pooled tissues from 2 to 3 mice (Foxp3fl/fl) were acquired on CytoFLEX LX (Beckman) and analyzed using Flowjo software (Tree Star).

**Proteomics profiling.** CD4+ YFP+ Treg cells were isolated from the peripheral lymph nodes and spleens of mice (8–12 weeks old) with indicated genotypes. To generate enough materials, the pooled tissues from 2 to 3 mice (Foxp3fl/fl, 8 to 11 (Foxp3fl/fl:Rbx1fl/fl) or 3 to 4 (Foxp3fl/fl:Ube2mfl/fl) were used. RNA samples were prepared with the miRNeasy Mini Kit (Qiagen, 21704), then reverse transcribed, amplified, labeled (Affymetrix GeneChip pico kit, 703308), and hybridized to Clariom S Arrays, mouse (Affymetrix, 902931), performed by Cnkingbio Biotech Co., Ltd. (Beijing, China). Microarray data sets were analyzed with Applied Systems Expression Console Software 1.4. Three independent experiments were performed.
Fig. 9 Rbx1 acts via Ube2m-dependent and -independent mechanisms in Treg cells. a Unbiased cluster analysis of the transcriptional programs revealed four categories of genes differentially expression in Rbx1- and Ube2m-deficient Treg cells, as compared to the wt control Treg cells. b Working model of Rbx1 and Ube2m in functional regulation of Treg cells. The Treg specific Rbx1 deletion causes the alterations in Treg functional genes, and multiple inflammatory and signaling, leading to early-onset immune disorder and death, whereas Ube2m deletion in Treg cells also caused altered inflammatory signaling pathway with much less extent, and death at the later stage. Lack of phenotype by deletion of Rbx2/Sag or Ube2f in Treg cells excludes the possible involvement of CRL5 in Treg regulation.
RNA and immunoblot analysis. The sequences of the primers pairs used in qPCR are as follows: AGTGGGAGTTGCG-3′/AGATGG-3′; and anti-β-Actin, HuaBio, M1210-2 (dilution 1: 5000).

Statistical analysis. The p values were calculated using Mann–Whitney test, two-sided unpaired Student’s t-test, one-way ANOVA or two-way ANOVA as indicated using GraphPad Prism, unless otherwise noted. Statistical analysis of mouse survival and respective p values were determined using the log-rank test. p < 0.05 was considered as significant. All error bars represent the SEM from three independent experiments.

Data availability. The scRNA-seq and microarray data generated in this study have been deposited in the Gene Expression Omnibus under accession code GSE199289 and GSE192664. The mass spectrometry proteomics data generated in this study have been deposited in the ProteomeXchange Consortium via the iProX partner repository under accession code PXD003144. Source data are provided with this paper.

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References
1. Zhao, Y. & Sun, Y. Cullin-RING ligases as attractive anti-cancer targets. Curr. Pharm. Des. 19, 3215–3225 (2013).
2. Huang, D. T. et al. E2-RING expansion of the NEDD8 cascade confers specificity to Cullin modification. Mol. Cell 33, 483–495 (2009).
3. Soucy, T. A. et al. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature 458, 732–736 (2009).
4. Nakayama, K. I. & Nakayama, K. Ubiquitin ligases: Cell-cycle control and cancer. Nat. Rev. Cancer 6, 369–381 (2006).
5. Zhou, W., Wei, W. & Sun, Y. Genetically engineered mouse models for functional studies of SKP1-CUL1-F-box-protein (SCF) E3 ubiquitin ligases. Cell Res. 23, 599–619 (2013).
6. Zhou, L., Zhang, W., Sun, Y. & Jia, L. Protein neddylation and its alterations in cancer. Nat. Immunol. 11, 106–116 (2010).
7. Tan, M., Davis, S. W., Saunders, T. L., Zhu, Y. & Sun, Y. RBX1/ROC1 partially rescues by simultaneous loss of p27. Nat. Rev. Cancer 10, 106–116 (2010).
8. Hu, W. et al. Regulatory T cells function in established systemic inflammation and reverse fatal autoimmunity. Nat. Immunol. 22, 1163–1174 (2021).
9. Delgoffe, G. M.et al. Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. Nat. Immunol. 256 (2013).
10. Herman, A. E., Freeman, G. J., Mathis, D. & Benoist, C. CD4+ regulatory cells that control intestinal inflammation are functionally active and regulatory T cell differentiation and function. Nat. Rev. Immunol. 16, 220–233 (2016).
11. Joetham, A. et al. Naturally occurring lung CD4+CD25+ T reg cells are potent inhibitors of GVHD and BM graft rejection. Blood 108, 3804–3812 (2004).
12. Tischner, D. et al. Defective cell death signalling along the Bcl-2 regulated apoptosis pathway compromises Treg cell development and limits their functionality in mice. J. Autoimmun. 38, 59–69 (2012).
13. Read, S., Malmstrom, V. & Powrie, F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25+CD4+ regulatory cells that control intestinal inflammation. J. Exp. Med. 192, 295–302 (2000).
14. Hu, W. et al. Regulatory T cells function in established systemic inflammation and reverse fatal autoimmunity. Nat. Immunol. 22, 1163–1174 (2021).
15. Zucchi, S. et al. Paracrine effect of regulatory T cells promotes cardiomyocyte proliferation during pregnancy and after myocardial infarction. Nat. Commun. 26, 2432 (2018).
16. Chinen, T. et al. An essential role for the IL-2 receptor in Treg cell function. Nat. Immunol. 17, 1322–1333 (2016).
17. Delgado, S. et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J. Exp. Med. 204, 1257–1265 (2007).
18. Borsellino, G. et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: Hydrolysis of extracellular ATP and immune suppression. Blood 110, 1225–1232 (2007).
19. Gronoff, G. M. et al. Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. Nat. Medicine 51, 252–256 (2016).
20. Huang, C. T. et al. Role of LAG-3 in regulatory T cells. Immunity 21, 503–513 (2004).
21. Venet, F. et al. Human CD4+CD25+ regulatory T lymphocytes inhibit lipopolysaccharide-induced monocyte survival through a Fas/Fas ligand-dependent mechanism. J. Immunol. 177, 6540–6547 (2006).
22. Garin, M. I. et al. Galactin-1: A key effector of regulation mediated by CD4+CD25+ T cells. Blood 109, 2058–2065 (2007).
47. Zhang, C. et al. Cullin3-KLHL25 Ubiquitin ligase targets ACLY for degradation to inhibit lipid synthesis and tumor progression. Genes Dev. 30, 1956–1970 (2016).

48. Ambrosini, G., Seelman, S. L. & Schwartz, G. K. Differentiation-related gene-1 decreases Bim stability by proteasome-mediated degradation. Cancer Res. 65, 6115–6121 (2009).

49. Lu, G. et al. Phosphorylation of ETS1 by Src family kinases prevents its recognition by the COP1 tumor suppressor. Cancer Cell 26, 222–234 (2014).

50. Nakayama, K. et al. Impaired degradation of inhibitory subunit of NF-kappa B (I Kappa B) and Beta-Catenin as a result of targeted disruption of the beta-TcP1 gene. Proc. Natl Acad. Sci. USA 100, 8752–8757 (2003).

51. Zhang, J. et al. VHL substrate transcription factor ZHX2 as an oncogenic driver in clear cell renal cell carcinoma. Science 3699, 290–295 (2018).

52. Huang, H. et al. Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. Proc. Natl Acad. Sci. USA 102, 1649–1654 (2005).

53. Yang, K. et al. Homeostatic control of metabolic and functional fitness of Treg cells by LKB1 signalling. Nature 548, 602–606 (2017).

54. O’Connor, L. et al. Bim: A novel member of the Bcl-2 family that promotes apoptosis. EMBO J. 17, 384–395 (1998).

55. Chougnet, C. A. et al. A major role for Bim in regulatory T cell homeostasis. J. Immunol. 186, 156–163 (2011).

56. Zhao, Y., Morgan, M. A. & Sun, Y. Targeting Neddylation pathways to inactivate cullin-RING ligases for anticancer therapy. Antioxid. Redox Signal. 21, 2383–2400 (2014).

57. Deshaies, R. J. SCF and Cullin/Ring H2-based ubiquitin ligases. Annu. Rev. Cell Dev. Biol. 15, 435–467 (1999).

58. Ouyang, W. et al. Novel Foxo1-dependent transcriptional programs control Treg cell function. Nature 491, 584–593 (2012).

59. Zeng, H. et al. mTORC1 couples immune signals and metabolic programming to establish Treg cell function. Nature 516, 482–488 (2015).

60. Han, X. et al. Mapping the mouse cell atlas by microwell-seq. Cell 166, 219–236 (2016).

61. Weinberg, S. E. et al. Mitochondrial complex III is essential for suppressive function of regulatory T cells. Nature 565, 495–499 (2019).

62. Su, W. et al. Protein Prenylation Drives Discrete Signaling Programs for the Differentiation and Maintenance of Effector Treg Cells. Cell Metab. 32, 996–1011 (2020).

63. Ivan, M. et al. HiAlpha targeted for VHL-mediated destruction by proline hydroxylation: Implications for O2 sensing. Science 292, 464–468 (2001).

64. Wang, D. et al. A deep proteome and transcriptome abundance atlas of 29 healthy human tissues. Mol. Syst. Biol. 15, e8503 (2019).

65. Jiang, L. et al. A quantitative proteome map of the human body. Cell 20, 31078–31083 (2020).

66. Cuadrado, E. et al. Proteomic analyses of human regulatory T cells reveal adaptations in signaling pathways that protect cellular identity. J. Immunol. 182, 3131–3138 (2009).

67. Tong, J. et al. m6A mRNA methylation sustains Treg suppressive functions. Cell Res. 28, 253–258 (2018).

68. Zhang, M. et al. The roles of Egr-2 in autoimmune diseases. Inflammation 38, 972–977 (2015).

69. Ilelem, A. et al. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4⁺CD25⁺ regulatory T cells. J. Exp. Med. 194, 847–853 (2001).

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Author contributions

D.W. designed and performed experiments, analyzed data, and wrote the manuscript. H.L. performed the proteome and transcriptome data. M.L. performed the mass spectrum experiment and analyzed the data. Y.S. designed experiments, analyzed data, wrote and finalized the manuscript, and oversaw the project.

Competing interests

The authors declare no competing interests.

Additional information

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