Genome-wide fitness gene identification reveals Roquin as a potent suppressor of CD8 T cell expansion and anti-tumor immunity

Highlights

- Genome-wide identification of CD8 T cell fitness genes in vitro and in vivo
- Expansion of CD8 T cells in vivo requires many more genes than in vitro expansion
- Roquin is a potent repressor of CD8 T cell expansion and anti-tumor immunity
- Roquin target IRF4 overexpression boosts CD8 T cell expansion and anti-tumor immunity

In brief

A canonical feature of adaptive immunity is clonal expansion. Through genome-wide CRISPR screens in vitro and in vivo, Zhao et al. systematically identify genes that modulate CD8 T cell expansion and reveal a key role of the Roquin-IRF4 axis in T cell expansion and anti-tumor immunity.
Genome-wide fitness gene identification reveals Roquin as a potent suppressor of CD8 T cell expansion and anti-tumor immunity

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SUMMARY

Robust expansion of adoptively transferred T cells is a prerequisite for effective cancer immunotherapy, but how many genes in the genome modulate T cell expansion remains unknown. Here, we perform in vivo and in vitro CRISPR screens to systematically identify genes influencing CD8 T cell expansion. In the mouse genome, ~2,600 and ~1,500 genes are required for optimal CD8 T cell expansion in vivo and in vitro, respectively. In vivo-specific CD8 T cell essential genes are enriched in metabolic pathways, including mitochondrial metabolism. The strongest repressor of CD8 T cell expansion is Roquin, the ablation of which drastically boosts T cell proliferation by enhancing cell-cycle progression and upregulation of IRF4. Roquin deficiency or IRF4 overexpression potently enhances anti-tumor immunity. These data provide a functional catalog of CD8 T cell fitness genes and suggest that targeting the Roquin-IRF4 axis is an effective strategy to enhance efficacy of adoptive transfer therapy for cancer.

INTRODUCTION

CD8 T cells play central roles in fighting against infection and cancer (Zhang and Bevan, 2011). A fundamental feature of T cell response is clonal expansion of antigen-specific naive T cells. The number of naive T cells specific for any given epitope is extremely low (Blattman et al., 2002; Obar et al., 2008), which demands these cells go through extensive clonal expansion to reach enough numbers for efficient elimination of target cells. Activated CD8 T cells divide every 4–6 h (Yoon et al., 2010; Zhang and Bevan, 2011), which are among the fastest proliferating cell types in mammals (Williams and Bevan, 2007; Zhang and Bevan, 2011). Upon activation, the transition of naive CD8 T cells from a quiescent state to a highly proliferative state is driven by an integrated program with comprehensive epigenetic, transcriptional, and metabolic rewiring (Buck et al., 2017; Kaech and Cui, 2012; Maclver et al., 2013; O’Neill et al., 2016). With decades of studies, mainly by mouse genetics, we have understood basic principles of T cell expansion and developed effective immunotherapies based on these principles, including adoptive T cell transfer therapy (ACT) of cancer (Guedan et al., 2019; Restifo et al., 2012). However, it remains unknown how many genes in the genome are actively involved in CD8 T cell expansion. Further dissection of the mechanisms of CD8 T cell expansion will not only deepen our understanding of this complex biological process but also generate targets for immunotherapy.

Robust expansion of adoptively transferred T cells in vivo is a prerequisite of effective ACT in patients (Lee et al., 2015; Nee-lapu et al., 2017). For reasons still not fully understood, in vitro-activated T cells expand poorly after transfer into lymphoreplete host (Gattinoni et al., 2006; Overwijk et al., 2003). In both mouse models and human patients, ACT is usually preceded by lymphodepleting conditioning procedures, such as cytotoxic drug treatment (e.g., fludarabine and/or cyclophosphamide) or total body irradiation, which is critical for the expansion of transferred T cells and therapeutic efficacy (Gattinoni et al., 2006). However, lymphodepleting chemotherapy causes various side effects, which can be life-threatening (Dudley et al., 2005, 2008; Lowe et al., 2018; Muranski et al., 2006). Thus, conditioning-free ACT remains to be developed.

The recent development of CRISPR/Cas9-based genetic screening provides a powerful platform to dissect gene function in a high-throughput manner. Initial genome-wide CRISPR screens in T cells performed in vitro demonstrated the feasibility of pooled screens in primary T cells (Henriksson et al., 2019; Shi-fruit et al., 2018). Due to coverage issues, most in vivo CRISPR screens performed in T cells in later studies used libraries focused on transcription factors or metabolism (Chen et al., 2021; Huang et al., 2021; Wei et al., 2019). These studies were
optimized for positive selections (enrichment of cells), and few novel essential genes were discovered, suggesting drop-out screens in vivo are still difficult due to random loss of cells. One study performed genome-wide CRISPR screens in T cells in vivo using the lymphopenic Rag1-1-knockout mice as recipients (Dong et al., 2019). Lymphopenia is known to drive T cell expansion; thus, T cell expansion in immunodeficient mice is different from that of lymphoreplete mice. These studies indicate that genome-wide CRISPR screens in T cells in vivo in fully lymphoreplete host remains challenging, especially for drop-out screens that require full coverage to exclude random loss of cells.

Here, to systematically identify genes that modulate the expansion of adoptively transferred T cells in lymphoreplete host, we performed genome-wide CRISPR screens in CD8 T cells in immunocompetent mice. We also performed in vitro screens in parallel to compare the sensitivity and potency between in vivo and in vitro screens. Most, if not all, genes involved in basic cellular processes (e.g., DNA replication, transcription, and translation) were recovered in our screens as essential genes, demonstrating genome-wide drop-out screening in primary T cells is feasible. Importantly, previously unrecognized genes essential for CD8 T cell expansion were discovered, including 1110004E09Rik and Gm10406, with unknown biological functions. By comparing screening results from in vivo and in vitro, we found that many more genes were required for optimal expansion of CD8 T cells in vivo than that in vitro. Many of these in vivo-specific essential genes were enriched in metabolic pathways, including energy metabolism.

In contrast with thousands of genes required for the optimal expansion of adoptively transferred CD8 T cells, the absence of only a few genes could potently boost CD8 T cell expansion. These genes include known tumor suppressors Trp53 and Pten, as well as genes reported by recent studies, such as Ptpn2 and Zc3h12a (LaFleur et al., 2019; Wei et al., 2019). Unexpectedly, the top gene repressing CD8 T cell expansion from our in vivo screening was Rc3h1. Rc3h1 encodes the RNA-binding protein Roquin that regulates inflammation (Vinuesa et al., 2005), but little is known about its role in CD8 T cells. Ablation of Roquin drastically boosted the expansion of adoptively transferred CD8 T cells and conferred much better control of tumor growth in fully immunocompetent mice without lymphodepletion conditioning, supplementation of cytokines, or vaccination. Mechanistically, Roquin repressed genes involved in DNA replication, and its absence promoted DNA synthesis and cell-cycle progression. From transcriptomic analysis, we found that interferon regulatory factor 4 (IRF4) was a key functional target downstream of Roquin. Expression of IRF4 was increased in Roquin-deficient CD8 T cells, and co-deletion of IRF4 completely abrogated the expansion-promoting effect of Roquin deficiency. Furthermore, overexpression of IRF4 was sufficient to boost the expansion of adoptively transferred CD8 T cells in immunocompetent mice and potently enhanced anti-tumor immunity.

Together, our genome-wide CRISPR screens in vitro and in vivo in immunocompetent mice generated a functional catalog of CD8 T cell fitness genes in the mouse genome. These data uncover previously unknown essential genes of CD8 T cells, reveal in vivo-specific essential genes in metabolic pathways, and demonstrate that the Roquin-IRF4 axis is a potent suppressor of CD8 T cell expansion and anti-tumor immunity, which may be targeted for immunotherapies.

**RESULTS**

**Genome-wide identification of CD8 T cell fitness genes in vivo and in vitro**

We constructed a retroviral vector expressing single guide RNA (sgRNA) with a Thy1.1 marker (Figure S1A), which rapidly and efficiently inactivated gene expression after delivery into Cas9-expressing T cells (Figure S1B). A genome-wide sgRNA library was cloned into this vector for screening in primary T cells (Figure S1C) (Doench et al., 2016). OT-I cells expressing Cas9 (Cas9;OT-I) were activated and infected with the retroviral sgRNA library, and cells expressing sgRNA were magnetically purified with anti-Thy1.1 nanobeads (Figure 1A). Purified cells were either expanded in vitro in the presence of IL-2 or transferred into wild-type mice for expansion in vivo without lymphodepletion or supplementation of cytokines (Figure 1A). On day 7 post-activation, cells expanded in vitro were collected, transferred T cells were isolated from spleens by flow cytometry sorting, and sgRNA distribution was analyzed by deep sequencing (Figure 1A).

With average depletion of more than 33% from 4 sgRNAs of each gene, we identified 2,642 and 1,555 essential genes that were required for optimal expansion of CD8 T cells in vivo and in vitro, respectively (Figure 1B; Tables S1, S2, and S3). These numbers of essential genes are at a similar range of essential genes reported in cancer cell lines (Behan et al., 2019; Hart et al., 2015; Wang et al., 2015), which vary depending on screening method, cut-off, and cell of origin, indicating activated CD8 T cells do not require significantly more genes to support rapid proliferation.

Known essential genes involved in fundamental cellular processes (e.g., DNA replication, transcription, and translation) were consistently depleted in both screens (Figure 1C). For example, every single component of ribosome was recovered as an essential gene from both in vitro and in vivo screens (Figure S1D), demonstrating genome-wide drop-out screens in primary T cells can be achieved in vivo as efficiently as in vitro. With this kind of potency, the false-negative rates of our screens are low (Tables S1 and S2). To test this, we selected two genes with unknown functions in T cells, namely, 1110004E09Rik and Gm10406 (Figures 1D and 1E; Tables S1 and S2). Ablation of either 1110004E09Rik or Gm10406 significantly impaired the expansion of CD8 T cells (Figures 1F and 1G), validating these two genes are bona fide essential genes of CD8 T cells. Because there is limited information available for 1110004E09Rik and Gm10406 in the literature, future studies are warranted to explore how these two genes contribute to CD8 T cell expansion. These validation data indicate our fitness gene lists for CD8 T cells have high confidence, which may serve as a reference for the community.

**In vivo-specific CD8 T cell essential genes are enriched in metabolic pathways**

Our screening results revealed that significantly more genes were required for optimal expansion of CD8 T cells in vivo than...
Figure 1. Genome-wide T cell fitness gene screens identify known and unknown essential genes of CD8 T cells

(A) Experimental design of genome-wide CRISPR screening of CD8 T cell fitness genes in vivo and in vitro.

(B) Numbers of CD8 T cell essential genes from in vivo and in vitro screens (depletion > 33%).

(C) KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis of shared essential genes from in vivo and in vitro screens.

(D and E) Distribution of sgRNA fold change (FC) and p value from in vivo (D) and in vitro (E) screens (FC < 1). Selected genes are labeled.

(F and G) Validation of essential genes (1110004E09Rik and Gm10406) of CD8 T cells. Activated Cas9;OT-I cells were infected with retrovirus expressing indicated sgRNAs and expanded in vitro in the presence of IL-2. The percentage of Thy1.1+ among CD8+ cells was monitored daily. Flow cytometry plots (F) and statistical analysis (G, n = 3) are shown. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, two-tailed unpaired Student’s t test. Data are representative of three independent experiments (mean ± SEM).

NS, not significant. See also Figure S1.
that in vitro (Figure 1B; Table S3). Gene set enrichment analysis (GSEA) showed that in vivo-specific essential genes of CD8 T cells were enriched in metabolic pathways and major histocompatibility complex class I and V were shown. A plot of in vivo-specific essential genes of CD8 T cells. Selected genes in MHC class I processing and ETC are labeled. Validation of the differential requirement of Ndufa2 and Atp5b for CD8 T cell expansion in vitro and in vivo. (C-E) Experimental design. Activated Cas9;OT-I cells were infected with retrovirus expressing indicated sgRNAs. Cells were either expanded in vitro with IL-2 or an equal number of CD8 Thy1.1+ cells were transferred into wild-type mice for in vivo expansion. The percentage of Thy1.1+ among CD8 Thy1.1+ cells from in vitro culture and spleens (in vivo expansion) was examined on day 7 post-activation. Flow cytometry plots (D) and summary data (E) are shown. ***p < 0.001, ****p < 0.0001, two-tailed unpaired Student’s t test. Data are representative of two independent experiments (mean ± SEM). See also Figure S2.
To validate these data, we selected two genes with differential requirement for CD8 T cell expansion between in vivo and in vitro, the complex I component Ndula2 and complex V component Atp5b (Figures 2C, S2C, and S2G). Consistent with our screening data, both Ndula2 and Atp5b were dispensable for CD8 T cell expansion in vitro but essential for CD8 T cell expansion in vivo (Figures 2D and 2E). These data again support that the false discovery rates of our screening system are low (Tables S1 and S2).

Together, these data demonstrate that more genes are required for optimal expansion of CD8 T cells in vivo than that in vitro, and some in vivo-specific fitness genes are enriched in metabolic regulation.

Roquin is one of the few genes potently repressing CD8 T cell expansion in vivo

We then focused on genes whose absence potently promoted CD8 T cell expansion in immunocompetent mice. Known tumor suppressors Trp53 and Pten were among top hits suppressing CD8 T cell expansion, confirming fidelity of our screening. However, unlike 10- to 100-fold enrichment of negative regulators of T cell expansion in focused screens (Chen et al., 2021; Wei et al., 2019), even these strong tumor suppressors showed only 2- to 3-fold enrichment in our genome-wide screens (Figures 3A and 3B; Tables S1 and S2). This is likely due to large library size and/or low input cell number. Nevertheless, most reported negative regulators of T cell expansion were successfully recovered and ranked as top hits in our screens, including Socs1, Ptpn2, and Zc3h12a (Dudda et al., 2013; LaFleur et al., 2019; Wei et al., 2019).

Unexpectedly, Rc3h1 represented the top 1 and top 2 hits repressing CD8 T cell expansion from our in vivo and in vitro screens, respectively (Figures 3A and 3B). Roquin (Roquin1), encoded by Rc3h1, is an RNA-binding protein promoting RNA decay with few studies in CD8 T cells (Bertossi et al., 2011; Viernes et al., 2005). To validate the role of Roquin in CD8 T cell expansion, we transferred 0.4 million Cas9;OT-I cells expressing Roquin-deficient OT-I cells into wild-type mice without lymphodepletion or cytokine supplement (Figures 3C and 3D). On day 7 post-activation when CD8 T cell response peaked, control Thy1.1+ OT-I cells were 20% of such cells were found in the absence of Roquin (Figures S4H–S4K). These data demonstrate that Roquin deficiency promotes CD8 T cell expansion without impairment of their functions.

To explore whether Roquin deficiency affected the memory potential of CD8 T cells, we analyzed the expression of CD127 and CD62L. Likely because of lack of antigen and inflammation in vivo in our system, the majority of control OT-I cells expressed high levels of CD127 and CD62L and barely expressed KLRG1 (Figures S3P–S3U). Although Roquin deficiency reduced the percentages of CD127+ and CD62L+ cells, the absolute numbers of these cells were in fact increased (Figures S3P–S3U), demonstrating that generation of more effector cells by Roquin deficiency is not at the cost of losing memory precursors.

Ablation of Roquin in CD8 T cells enhances anti-tumor immunity in immunocompetent mice without conditioning

Except for those performed in immunodeficient models (e.g., Rag1/2-deficient mice or NSG mice), ACT of tumor in immunocompetent models is usually preceded by lymphodepletion or total body irradiation that enhances the expansion of transferred T cells (Gattinoni et al., 2006). To test whether Roquin deficiency could implement conditioning-free ACT of tumor, we transferred control or Roquin-deficient OT-I cells into wild-type mice bearing EL4-OVA lymphoma without conditioning, supplement of cytokine, or vaccination (Figure 4A). Transfer of control cells without conditioning showed mild repression of tumor growth and marginally extended the survival of tumor-bearing mice, which is consistent with published data (Oenvijk et al., 2003). In contrast, Roquin-deficient OT-I cells conferred much better tumor control and significantly prolonged mouse survival (Figures 4B–4D).

Analysis of tumor-infiltrating lymphocytes (TILs) showed that Roquin deficiency significantly increased the infiltration of transferred T cells into tumors (Figures 4E and 4F). The expression of inhibitory receptors, including PD-1, TIM-3, and LAG-3, on TILs was comparable or slightly reduced in Roquin-deficient OT-I cells (Figures S4A–S4G). IFN-γ and TNF-α productions were also comparable between control and Roquin-deficient OT-I cells (Figures S4H–S4K). These data demonstrate that Roquin deficiency promotes anti-tumor immunity primarily by boosting CD8 T cell expansion.

Roquin inhibits DNA replication and cell-cycle progression of CD8 T cells

To explore how Roquin deficiency boosts T cell expansion, we analyzed differentially expressed genes between control and Roquin-deficient CD8 T cells isolated from spleens (Figure 5A). RNA sequencing (RNA-seq) data showed that 2,185 genes were upregulated, while 1,175 genes were downregulated, in Roquin-deficient OT-I cells compared with control cells (Figure 5B). The higher number of upregulated genes is consistent with Roquin as an RNA-binding protein promoting mRNA decay.
Akira and Maeda, 2021; Fu and Blackshear, 2017). GSEA showed that upregulated genes were highly enriched in cell cycle, E2F targets, G2M checkpoint, and mitotic spindle (Figure 5C). For example, positive regulators of cell cycle, such as E2f1, E2f2, and E2f3, were all upregulated, while inhibitors of cell-cycle progression, such as E2f4 and E2f5, were downregulated (Figure S5A). In addition, mRNA encoding proteins directly responsible for DNA replication were also upregulated (Figure S5B), including primer-synthesizing enzymes Prim1 and Prim2, and replication helicase components Mcm2–Mcm7, DNA polymerases Pola1 and Pole, and DNA ligase Lig1 (Burgers and Kunkel, 2017). The upregulation of selected genes involved

Figure 3. Roquin is a potent suppressor of CD8 T cell expansion
(A and B) Plots show the ranking of all genes from in vivo (A) and in vitro (B) screens based on sgRNA enrichment and depletion. Top hits of enriched genes are labeled.
(C) Experimental design for validation of Roquin regulation of CD8 T cell expansion. Activated Cas9;OT-I cells were infected with retrovirus expressing non-targeting sgRNA (sgControl) or sgRNA targeting Rc3h1 (sgRc3h1), and an equal number of CD8+Thy1.1+ cells were transferred into wild-type mice. The percentage of Thy1.1+ among CD8+ cells in blood was monitored by flow cytometry from day 5 to day 10 post-activation.
(D) Immunoblots of Roquin, Roquin2, and GAPDH from Cas9;OT-I cells expressing indicated sgRNAs.
(E and F) Experiments were performed as in (C). Flow cytometry plots (E) and statistical analysis (F, n = 3) of Thy1.1+ among CD8+ cells from peripheral blood are shown.
(G–I) Mice were killed on day 8 after activation (day 5 post-transfer), and splenocytes were analyzed. Flow cytometry plots (G) and statistical analysis (H and I, n = 4) are shown.

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, two-tailed unpaired Student’s t test. Data are representative of three independent experiments (mean ± SEM). See also Figure S3.
in DNA replication and cell cycle was validated individually by quantitative polymerase chain reaction (qPCR) (Figure S5C). These data indicate Roquin deficiency in CD8 T cells promotes DNA replication and cell-cycle progression.

Roquin is known to promote the decay of mRNAs encoding proteins involved in immune regulation, such as ICOS, CTLA4, and cRel (Jeltsch et al., 2014), but it has not been reported to regulate mRNA involved in cell cycle and DNA replication. We examined the half-life of mRNAs encoding proteins responsible for DNA replication with ICOS as a positive control. As reported previously (Jeltsch et al., 2014), decay of Icos mRNA after actinomycin D treatment was slowed down in the absence of Roquin (Figure 5D). Similarly, decay of mRNAs of Pola1, Prim1, and Prim2 was also slowed down in Roquin-deficient CD8 T cells (Figure 5D), suggesting Roquin modulates the stability of these mRNAs.

We then analyzed the influence of Roquin deficiency on cell cycle by 5-ethynyl-2'-deoxyuridine (EdU) labeling. On day 6 after activation (day 4 post-transfer), the percentage of CD8 T cells in S phase was significantly higher in the absence of Roquin (Figures 5E and 5F). Consistently, the level of proliferation marker Ki-67 was also higher in Roquin-deficient CD8 T cells compared with control cells (Figures 5G and 5H). In addition, Roquin deficiency did not affect CD8 T cell apoptosis (Figures S5D and S5E). Together, these data demonstrate that Roquin inhibits DNA replication and cell-cycle progression of adoptively transferred CD8 T cells, and its absence boosts T cell proliferation.

**IRF4 is a functional target of Roquin in CD8 T cells**

To further dissect the mechanism underlying Roquin regulation of CD8 T cell expansion, we focused on direct targets of Roquin. ICOS is an important target of Roquin, whose upregulation has been reported to be responsible for the phenotype of a germline Roquin mutation mouse strain (Yu et al., 2007). Indeed, ICOS protein level was upregulated in Roquin-deficient CD8 T cells compared with that of control cells (Figures S6A and S6B). However, inhibition of ICOS expression in Roquin-deficient CD8 T cells did not compromise the expansion-promoting effect of Roquin deficiency (Figures S6A–S6D). In addition, overexpression of ICOS was not able to boost CD8 T cell expansion (Figures S6E–S6H). These data demonstrate that increased ICOS expression does not contribute to the rapid expansion of Roquin-deficient CD8 T cells.
RNA-seq analysis showed several transcription factors involved in T cell activation and/or proliferation were upregulated in the Roquin-deficient CD8 T cells, including IRF4 (Figure 6A). IRF4 is a key transcription factor promoting the activation, proliferation, and metabolic reprogramming of activated T cells (Man et al., 2013; Mittrücker et al., 1997; Yao et al., 2013), which is also a direct target of Roquin (Jeltsch et al., 2014). We validated that IRF4 protein level was increased in Roquin-deficient CD8 T cells (Figures 6B and 6C). Co-deletion of IRF4 with Roquin completely reversed the effect of Roquin deficiency in CD8 T cell expansion.
Figure 6. IRF4 is a key functional target of Roquin regulating CD8 T cell expansion

(A) A heatmap of differentially expressed transcription factors between control and Roquin-deficient CD8 T cells. (B and C) Increased IRF4 protein level in Roquin-deficient CD8 T cells. Activated Cas9;OT-I cells were infected with retrovirus expressing indicated sgRNAs and transferred into wild-type mice. On day 3 post-transfer, IRF4 expression level of CD8+Thy1.1+ cells from spleen of mice was examined. Flow cytometry plots (B) and statistical analysis (C, n = 4) are shown. (D–F) Co-deletion of IRF4 nullifies the expansion-promoting effect of Roquin deficiency in CD8 T cells. Five million Cas9;OT-I cells expressing indicated sgRNAs were transferred into wild-type mice. On day 5 after transfer, mice were killed and CD8+Thy1.1+ cells from spleens were examined. Flow cytometry plots (D), percentage (E, n = 4), and absolute cell number (F, n = 4) are shown. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, two-tailed unpaired Student’s t test. Data are representative of two independent experiments (mean ± SEM). See also Figure S6.

(Figures 6D–6F), demonstrating that IRF4 is a functional target of Roquin in CD8 T cell expansion.

Together, these data demonstrate that, unlike in CD4 T cells (Yu et al., 2007), upregulation of ICOS is dispensable for Roquin to regulate CD8 T cells. Instead, increased expression of IRF4 is essential for the rapid expansion of Roquin-deficient CD8 T cells.

**Overexpression of IRF4 boosts CD8 T cell expansion and anti-tumor immunity**

Although IRF4 was required for CD8 T cell expansion boosted by Roquin deficiency, it was unknown whether overexpression of IRF4 is sufficient to promote CD8 T cell expansion. To test this, we transduced activated OT-I cells with retrovirus expressing IRF4 (pMIG-Irf4-IRES-Thy1.1) or the empty vector (pMIG-IRES-Thy1.1) and transferred cells into immunocompetent mice to test their expansion in vivo (Figure 7A). On day 5 after transfer (day 8 post-activation), there were ~10-fold more OT-I cells overexpressing IRF4 in spleens compared with that of control (Figures 7B–7D, S7A, and S7B). Cell-cycle analysis showed that IRF4 overexpression increased the percentage of cells in S phase (Figures S7C and S7D). Consistently, the level of Ki-67 was also increased in IRF4-overexpressing CD8 T cells (Figures S7E and S7F). These data demonstrate that IRF4 overexpression boosts CD8 T cell expansion by promoting cell proliferation, like Roquin deficiency.

To test whether IRF4 overexpression could enhance anti-tumor immunity in ACT, we adoptively transferred OT-I cells overexpressing IRF4 protein or control vector into mice bearing EL4-OVA tumors (Figure 7E). Although transfer of control OT-I cells expressing empty vector showed negligible effects on tumor growth and survival, OT-I cells overexpressing IRF4 exhibited potent tumor killing and significantly prolonged the survival of mice without conditioning in a fully immunocompetent setting (Figures 7F–7H). Together, these data demonstrate that overexpression of IRF4 boosts the expansion of adoptively transferred CD8 T cells and enhances anti-tumor immunity.

**DISCUSSION**

Clonal expansion is a canonical feature of adaptive immunity. In this study, we systematically characterized genes that modulate the expansion of adoptively transferred CD8 T cells in the mouse genome. Through genome-wide fitness gene characterization in primary cells in vivo with an immunocompetent and undisturbed environment, we successfully recovered known essential genes of mammalian cells, discovered previously unknown essential genes of CD8 T cells, and found Roquin as a potent repressor of CD8 T cell expansion and anti-tumor immunity.

Our side-by-side comparison of CD8 T cell fitness genes in vivo and in vitro showed that genes involved in fundamental cellular processes, such as DNA, RNA, and protein synthesis, were required for CD8 T cell expansion both in vivo and in vitro, as expected. However, significantly more genes are required for optimal expansion of CD8 T cells in vivo (Figure 1B). Except for NK cell-mediated killing of CD8 T cells losing MHC class I components, a significant part of in vivo-specific genes was enriched in metabolic pathways, particularly energy metabolism. Mitochondrial metabolism plays different roles in different T cell
subsets. CD4 T cells use glycolysis and oxidative phosphorylation (OXPHOS) interchangeably to support cell expansion in vitro (Chang et al., 2013), and complex II component succinate dehydrogenase complex subunit C (SDHC) inhibits T helper 1 expansion during in vitro cultures (Bailis et al., 2019). Our screening data allowed us to interrogate the role of each component of ETC in CD8 T cell expansion. Complex II and complex III were essential for CD8 T cell expansion both in vitro and in vivo, which is different from CD4 T cells. In addition, certain components of complex I and most complex V components were dispensable for CD8 T cell expansion in vitro but essential for their expansion in vivo (Figures 2D and 2E). We ruled out that this was due to hypoxia or lower glucose level in vivo (data not shown). Other limiting nutrients, such as aspartate, may be involved (Bailis et al., 2019; Birsoy et al., 2015; Sullivan et al., 2015). These data demonstrate that the roles of mitochondrial metabolism in T cell expansion (and likely function) are highly context dependent, and each component of ETC has different roles that have to be studied individually.

Our screens revealed a limited number of genes repressing CD8 T cell expansion. Although PTPN2, Regnase-1, and SOCS1 were reported previously (Dudda et al., 2013; LaFleur et al., 2019; Wei et al., 2019), a role of Roquin in CD8 T cell expansion was a surprise. Although initial studies showed that Roquin germline mutation (sanroque) mice had strong inflammatory phenotype with spontaneous T cell activation and expansion (Vinueza et al., 2005), later studies with T cell-specific deletion of Roquin demonstrated that those phenotypes were largely.

Figure 7. Overexpression of IRF4 enhances CD8 T cell expansion and anti-tumor immunity

(A–D) Overexpression of IRF4 enhances CD8 T cell expansion. (A) Experimental design. Activated OT-I cells were infected with retrovirus overexpressing empty vector pMIG-IRE- Thy1.1 or pMIG-IRF4-IRE-Thy1.1, and an equal number of CD8+ Thy1.1 cells were transferred into wild-type mice. The percentage of Thy1.1+ among CD8+ cells in spleens was examined on day 8 after activation. Flow cytometry plots (B), percentage (C, n = 4), and absolute cell number (D, n = 4) are shown.

(E–H) Overexpression of IRF4 in CD8 T cells boosts anti-tumor immunity. (E) Experimental design for ACT of tumor. Wild-type mice were inoculated with 0.5 million EL4-OVA tumor cells on right flank subcutaneously. After 7 days, PBS or an equal number of OT-I cells expressing pMIG-IRE- Thy1.1 or pMIG-IRF4-IRE-Thy1.1 were transferred into tumor-bearing mice. Tumor size was measured every 3 days. (F) Tumor size of mice receiving indicated treatment. (G) Tumor size on day 19 after inoculation (12 days after transfer). (H) Survival curve of tumor-bearing mice with indicated treatment. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, two-tailed unpaired Student’s t test for (C), (D), and (G); log-rank (Mantel-Cox) test for (H). Data are representative of two independent experiments (mean ± SEM). See also Figure S7.
T cell extrinsic, and CD8 T cells did not increase (Bertossi et al., 2011). Thus, our data that Roquin-deficient CD8 T cell expanded 10- to 20-fold more than that of controls was unexpected. Interestingly, another potent repressor of CD8 T cell expansion, Regnase-1, also promotes mRNA decay like Roquin (Jeltsch et al., 2014; Matsushita et al., 2009), suggesting posttranscriptional regulation of gene expression plays critical roles in CD8 T cell expansion. However, the mechanisms of Roquin- and Regnase-1-mediated regulation of CD8 T cell expansion are different (Mino et al., 2015). Although Regnase-1 deficiency mainly sustained long-term survival of effector CD8 T cells (Wei et al., 2019), Roquin deficiency primarily boosted CD8 T cell proliferation without the influence of apoptosis (Figures 5E–5H, S5D, and S5E). Consistently, T cell-specific deletion of Regnase-1 resulted in fetal autoimmunity in mice (Uehata et al., 2013), while deletion of Roquin in T cells was largely inconsequential in mice (Bertossi et al., 2011).

A common feature of PTPN2, SOCS1, Regnase-1, and Roquin is that they regulate T cells in a passive way, either dephosphorylation of signaling molecules (PTPN2 and SOCS1) or slowdown of mRNA decay (Regnase-1 and Roquin). As brakes of immune response, these molecules dampen the events initiated by T cell receptor (TCR) signaling and restore immune homeostasis (Akira and Maeda, 2021; Fu and Blackshear, 2017). The fact that very few genes were found to potently repress CD8 T cell expansion from our as well as other screens and that most of these factors turned out to promote dephosphorylation and mRNA decay suggest that, unlike the highly coordinated T cell activation program involving thousands of genes, activated T cells may not have a dedicated program to shut down immunity actively.

Roquin’s targets are mainly involved in immune regulation (Akira and Maeda, 2021; Fu and Blackshear, 2017). The increase of halflife of mRNAs encoding Pola1, Prim1, and Prim2 in Roquin-deficient CD8 T cells suggests that Roquin also regulates decay of factors responsible for DNA replication, which directly promote cell-cycle progression. However, whether this regulation is direct or indirect requires further investigations. Roquin is downregulated upon T cell activation by Malt1-mediated cleavage (Jeltsch et al., 2014), suggesting enabling rapid DNA replication via depression of Roquin is a critical event downstream of TCR signaling.

ICOS is a critical target of Roquin to regulate immune homeostasis, the loss of which could largely rescue the autoimmune phenotypes of sanroque mice (Yu et al., 2007). However, our data demonstrated that ICOS was dispensable for Roquin to regulate CD8 T cell expansion (Figure 5E). Instead, we found IRF4, another target of Roquin (Jeltsch et al., 2014), was required for Roquin to regulate CD8 T cell expansion (Figure 6). Importantly, overexpression of IRF4 alone was sufficient to boost CD8 T cell expansion (Figure 7). Recently, two other related transcription factors, BATF and c-JUN, were also reported to promote CD8 T cell expansion upon overexpression (Lynn et al., 2019; Wei et al., 2019). Although BATF, JUN, and IRF4 formed a protein complex to bind composite site on DNA to regulate gene expression (Glasmacher et al., 2012; Li et al., 2012), the fact that overexpression of one of three such proteins alone is sufficient to boost CD8 T cell expansion suggests that these proteins may regulate each other.

Lymphodepleting preconditioning is an integral part of ACT of cancer (Muranski et al., 2006). With the advancement of CRISPR-mediating modification of T cells in clinic (Stadtmauer et al., 2020), modulating gene expression in T cells by genetic engineering may help implement conditioning-free ACT, because all conditioning procedures eventually have to influence T cells. Our studies showed that either deletion of Roquin or overexpression of its target IRF4 potently boosted CD8 T cell expansion and anti-tumor immunity in immunocompetent mice without conditioning, suggesting Roquin and IRF4 are potential targets to implement conditioning-free ACT in cancer.

In summary, our studies demonstrated that genome-wide CRISPR screening in T cells in vivo in immunocompetent mice is feasible for both positive and negative selections. Employing this platform, we generated a functional catalog of CD8 T cell fitness genes in the mouse genome. Known and uncharacterized essential genes were identified, and targets for conditioning-free ACT of cancer were discovered.

LIMITATION OF STUDY

It has been reported that precursor frequency affects the kinetics and magnitudes of T cell expansion (Obar et al., 2008). In our in vivo screening, ~7 million OT-I cells were transferred into mice for expansion, which may not reflect the expansion of low-frequency naïve T cells during endogenous immune response. Our in vivo T cell expansion protocol resembled ACT used in clinic, which requires transfer of large numbers of engineered T cells into patients (Lee et al., 2015; Neelapu et al., 2017). From our screens, there were hundreds of in vitro-specific essential genes required for CD8 T cells expansion in vitro, but not in vivo (Figure 1B), which we considered as “artifacts” of in vitro cultures. Thus, these genes, as well as the underlying mechanisms, were not analyzed and explored in this study. In addition, in-depth analysis of in vivo-specific essential genes other than those involved in mitochondrial metabolism warrants further investigations. Ablation of Roquin or overexpression of IRF4 potently boosted CD8 T cell expansion and anti-tumor immunity in immunocompetent mice, but whether Roquin deficiency and IRF4 overexpression also modulates the expansion of human T cells needs to be tested.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Mice
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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.110083.

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AUTHOR CONTRIBUTIONS

H.Z. designed and performed experiments and analyzed data. Y.L., L.W., and G.J. performed experiments. X.Z., J.X., G.Z., and Y.M. provided technical support. N.Y. analyzed data and supervised the project. M.P. conceived and supervised the project, analyzed data, and wrote the paper.

DECLARATION OF INTERESTS

A patent application has been filed based on the findings described in this study.

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### KEY RESOURCES TABLE

#### REAGENT or RESOURCE | SOURCE | IDENTIFIER
--- | --- | ---
**Antibodies**
DAPI | BioLegend | Cat#422801
LIVE/DEAD Fixable Near-IR Dead Cell Stain | Invitrogen | Cat#L34976
Biotin anti-mouse Thy1.1 (OX-7) | BioLegend | Cat#202510, RRID: AB_2201417
PE anti-mouse Thy1.1 (OX-7) | BioLegend | Cat#202524, RRID: AB_1595524
APC anti-mouse CD8α (53-6.7) | BioLegend | Cat#100712, RRID: AB_312751
eFluor 450 anti-mouse CD8α (53-6.7) | Invitrogen | Cat#48-0081-82, RRID: AB_1272198
PE anti-mouse CD8α (53-6.7) | BioLegend | Cat#100708, RRID: AB_312747
PE-Cy7 anti-mouse CD8α (53-6.7) | Invitrogen | Cat#25-0081-82, RRID: AB_469584
PE anti-mouse CD45.2 (104) | Invitrogen | Cat#12-0454-83, RRID: AB_465679
Brilliant Violet 785 anti-mouse CD45.1 (A20) | BioLegend | Cat#110743, RRID: AB_2563379
PE anti-mouse ICOS (C398.4A) | BioLegend | Cat#313508, RRID: AB_416332
FITC anti-mouse CD25 (PC61) | BioLegend | Cat#102006, RRID: AB_312855
FITC anti-mouse PD-1 (29F.1A12) | BioLegend | Cat#135214, RRID: AB_10680238
PE anti-mouse PD-1 (29F.1A12) | BioLegend | Cat#135206, RRID: AB_1877231
PE-Cy7 anti-mouse TIM-3 (RMT3-23) | Invitrogen | Cat#25-5870-82, RRID: AB_2573483
PerCP-eFluor 710 anti-mouse LAG-3 (C9B7W) | Invitrogen | Cat#46-2231-82, RRID: AB_11151334
PE-Cy7 anti-mouse CD62L (MEL-14) | Invitrogen | Cat#25-0621-82, RRID: AB_469633
APC anti-mouse KLRG1 (2F1) | BD PharMingen | Cat#561620, RRID: AB_10523219
PE anti-mouse CD127 (A7R34) | BioLegend | Cat#135010, RRID: AB_1937251
FITC anti-mouse IFNγ (XMG1.2) | BioLegend | Cat#505806, RRID: AB_315400
PE anti-mouse TNFα (MP6-XT22) | Invitrogen | Cat#12-7321-82, RRID: AB_466199
APC anti-mouse GZMB (GB11) | Invitrogen | Cat#GRB05, RRID: AB_2536539
FITC anti-mouse Ki-67 (SolA15) | Invitrogen | Cat#11-5698-82, RRID: AB_11151330
PE anti-mouse IRF4 (IRF4.3E4) | BioLegend | Cat#646403, RRID: AB_2563004
PE Streptavidin | BioLegend | Cat#405204
APC Streptavidin | BioLegend | Cat#405243
FITC Streptavidin | Invitrogen | Cat#11-4317-87
**Chemicals, peptides, and recombinant proteins**
OVA257-264 peptide (SIINFEKL) | Chinapeptides | Cat#138831-86-4
Actinomycin D | CST | Cat#15021
Recombinant human IL-2 | PeproTech | Cat#200-02-1000
Ionomycin | Biovision | Cat#1566
GolgiStop | BD | Cat#554724, RRID: AB_2869012
Polybrene | Sigma-Aldrich | Cat#H9268
**Critical commercial assays**
NEBuilder® HiFi DNA Assembly Master Mix | NEB | Cat#E2621S
Q5® High-Fidelity DNA Polymerase | NEB | Cat#M0491L
TIANamp Genomic DNA Kit | TIANGEN | Cat#DP304
RNAprep Pure Cell/Bacteria Kit | TIANGEN | Cat#DP430
TIANScript II RT Kit | TIANGEN | Cat#KR107
Talent qPCR PreMix (SYBR Green) | TIANGEN | Cat#FP209
Transcription Factor Staining Buffer kit | BD PharMingen | Cat#562574, RRID: AB_2869424

(Continued on next page)
| **REAGENT or RESOURCE** | **SOURCE** | **IDENTIFIER** |
|-------------------------|------------|----------------|
| Deposited data          |            |                |
| Data files for RNA-seq  | This paper | GEO: GSE186284 |
| Experimental models: Cell lines | | |
| Phoenix-ECO            | ATCC       | Cat#CRL-3214, RRID: CVCL_H717 |
| EL4                    | ATCC       | Cat#TIB-39, RRID: CVCL_0255 |
| Experimental models: Organisms/strains | | |
| C57BL/6                | The Jackson Laboratory | Cat#JAX:000664, RRID: IMSR_JAX:000664 |
| OT-I transgenic mice (C57BL/6) | The Jackson Laboratory | Cat#JAX:003831, RRID: IMSR_JAX:003831 |
| Rosa26-Cas9 knockin mice (C57BL/6) | The Jackson Laboratory | Cat#JAX:026430, RRID: IMSR_JAX:026430 |
| CD45.1 (C57BL/6)       | The Jackson Laboratory | Cat#JAX:002014, RRID: IMSR_JAX:002014 |
| Oligonucleotides        |            |                |
| sgNon-targeting (sgControl), TTCGCACGATTGCACCTTGG | Doench et al., 2016 | Addgene #73632 |
| sgRNA targeting Pdcd1, GACACACGGC | Doench et al., 2016 | Addgene #73632 |
| sgRNA targeting 1110004E09Rik, TGGCCCGCGTCTACAACGGG | Doench et al., 2016 | Addgene #73632 |
| sgRNA targeting Gm10406, CTGCTATAAGTTCTCTGTG | Doench et al., 2016 | Addgene #73632 |
| sgRNA targeting Ndufa2, TCTGATCCGC | Doench et al., 2016 | Addgene #73632 |
| sgRNA targeting Atp5b, CCCACCTAGCCACCACAT | Doench et al., 2016 | Addgene #73632 |
| sgRNA targeting Rc3h1, GGATATAGCTGAGACCTCG | Doench et al., 2016 | Addgene #73632 |
| sgRNA targeting Rc3h1(guiden#2), ACGTGCGTGTGCAGATCAT | Doench et al., 2016 | Addgene #73632 |
| sgRNA targeting Icos, AAATGAAACATCCTATGAT | Doench et al., 2016 | Addgene #73632 |
| sgRNA targeting Irf4, CAAGCAGGACTACAATCGTGTG | Doench et al., 2016 | Addgene #73632 |
| Next-generation sequencing (NGS) primer | This paper | N/A |
| NGS-F1: AATGATACGGCGACCACCCAGGTACCTACACTTTCCTACAGCAAGCTCTCCGATCTGTTATATATCTTTGGGAAAGGAGAAACAC | | |
| NGS-R1: CAAGCAGAAGACGGCATACGAGATAAATGAGTAATGACTGAGTTCCAGACGTGTGCTCTTCCGATCTCCGACTCCGCGTGCCACTTTTCAA | | |
| Recombinant DNA         |            |                |
| Mouse CRISPR Knockout Pooled Library (Brie) | Doench et al., 2016 | Addgene #73632 |
| pCL-Eco                | Addgene    | Addgene #12371 |
| pMSCV-sgNon-targeting-Thy1.1 | This paper | N/A |
| pMSCV-sgPdcd1-Thy1.1   | This paper | N/A |
| pMSCV-sg1110004E09Rik-Thy1.1 | This paper | N/A |
| pMSCV-sgGm10406-Thy1.1 | This paper | N/A |
| pMSCV-sgNdufa2-Thy1.1  | This paper | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for reagents should be directed to lead contact, Min Peng (pengmin@tsinghua.edu.cn). Plasmids and cell lines generated in this study will be made available upon request. We may require a payment and/or a completed materials transfer agreement in case there is potential for commercial application.

Materials availability
This study did not generate new unique reagents.

Data and code availability
- All screening data can be found in Tables S1, S2, and S3. RNA sequencing data are available via NCBI Gene Expression Omnibus (GEO) database (GEO: GSE186284).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
C57BL/6, CD45.1, OT-I and Cas9 transgenic mice were originally from The Jackson Laboratory and maintained under specific pathogen-free conditions at the Laboratory Animal Research Center of Tsinghua University (Beijing, China). These animal facilities are approved by Beijing Administration Office of Laboratory Animal. All animal works were approved by Institutional Animal Care and Use Committee (IACUC).

Culture of mouse primary T cells
Mouse primary T cells were cultured in T cell medium (TCM): RPMI1640 medium (GIBCO) supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine, 55 μM β-mercaptoethanol, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 ng/ml IL-2 in a humidified incubator at 37°C with 5% CO2 and passaged every 1 - 2 days at the density of 1 - 2 million cells/ml.

Adoptive T cell transfer
Single cell suspension were prepared from spleen and lymph nodes of Cas9-expressing and OT-I transgenic mice (Cas9;OT-I). Total cells were loaded with 1 μM ovalbumin peptide (OVA\textsubscript{257-264} SIINFEKL) in TCM for 1 hour, then washed and cultured in TCM for
activation of OT-I cells. Twenty-four hours after activation, viral transduction was performed by spin-infection with 2,000 g at 33°C for 2 hours in the presence of 16 μg/ml polybrene, followed by incubation for another 4 hours. Then, cells were washed and cultured in fresh TCM with IL-2. Twenty-four hours after spin-infection, the efficiency of transduction was determined by examination of reporter (Thy1.1 or GFP) positive cells by flow cytometry. Then cells were either expanded in vitro with IL-2 or transferred into mice via tail vein for in vivo expansion. The effects of gene knockout or overexpression on T cell phenotypes were monitored.

**Tumor model**

EL4-OVA cells (0.5 million) were subcutaneously injected into the right flank of wild-type C57BL/6 mice. Seven days after tumor inoculation, tumor-bearing mice were randomly divided into 3 groups, PBS, control or gene-modified OT-I cells (1 million for sgControl or sg2C3h1 OT-I cells; 5 million for pMIG-IRES-Thy1.1 or pMIG-Irf4-IRES-Thy1.1 expressing OT-I cells) were adaptively transferred into tumor-bearing mice via tail vein. Tumor size and mouse survival were recorded every 2-3 days. Tumor size was calculated by length × width. Mice bearing a tumor > 300 mm² were considered as the endpoint of experiment and euthanized. To examine tumor-infiltrating lymphocytes (TILs), tumors were explanted and cut into pieces, digested with 1 mg/ml collagenase IV and 200 μg/mL DNase I at 37°C for 45 min and grinded through a 70 μm strainer. Single-cell suspension of TIL samples were analyzed by a LSRFortessa cytometer (BD).

**METHOD DETAILS**

**Vector and library construction**

To generate a retroviral vector for expression of single guide RNA (sgRNA) together with a Thy1.1 marker, the hU6-sgRNA-EF1α-Cas9-P2A-puro expression cassette from lentiCRISPRv2 (Addgene #52961) was cloned into pMSCV backbone (Addgene #74056), then Cas9-P2A-puro was replaced by a Thy1.1 cassette. This reconstructed vector was named pMSCV-sgRNA-Thy1.1. The sgRNA part of Brie genome-wide sgRNA library (Addgene #73632) for mouse was PCR-amplified and ligated with pMSCV-sgRNA-Thy1.1 via Gibson assembly (NEB). The ligated product was precipitated, washed and electroporated into TOP10 bacteria which were sprayed of Brie genome-wide sgRNA library (Addgene #73632) using calcium phosphate precipitate mediated transfection. The viral supernatant was collected at 24, 36, 48, 60 and 72 hours post-transfection, filtered via 0.45 μm filters, aliquoted and frozen at −80°C. In vivo screens were performed 2 times independently (coverage > 160 x on day 7 in each screen) and in vitro screens were performed 3 times (coverage > 160 x on day 7 in each screen) independently.

**Cell lines**

To generate OVA-expressing EL4 cell line (EL4-OVA), EL4 cells were transduced with retrovirus expressing OVA, GFP positive cells were sorted and expanded. Phoenix-ECO (ATCC) and EL4-OVA cells were cultured in DMEM (GIBCO) containing 5% FBS, 2 mM glutamine,100 units/ml penicillin and 100 μg/ml streptomycin in the incubator.

**Retrovirus production and viral transduction**

Retroviruses were packaged by co-transfection of Phoenix-ECO cells with indicated plasmid and helper plasmid pCL-Eco (Addgene #12371) using calcium phosphate precipitate mediated transfection. The viral supernatant was collected at 24, 36, 48, 60 and 72 hours post-transfection, filtered via 0.45 μM filters, aliquoted and frozen at −80°C.

**Genome-wide CRISPR screens of T cell fitness genes**

To perform expansion-based genome-wide in vivo and in vitro CRISPR screens, 300 - 450 million Cas9;OT-I were loaded with OVA257-264 peptide for activation as described above. Lib#7 viral transduction was performed as described above with MOI ~0.5, attaining a coverage of > 500 cells per sgRNA. Transduced cells were purified by biotin anti-mouse Thy1.1 antibody followed by streptavidin magnetic nanobeads (BioLegend). Thirty-six hours after transduction, 50 million cells were frozen at −80°C as input (> 500 cells per sgRNA). For in vivo screen, ~200 million cells were intravenously transferred into ~30 C57BL/6 mice. Four days post-transfer, Thy1.1+ splenocytes were sorted using a S3e cell sorter and frozen at −80°C. For in vitro screen, ~100 million cells were passaged in the presence of IL-2 until 6 days after transduction, then the cells were harvested and frozen at −80°C. In vivo screens were performed 2 times independently (coverage > 160 x on day 7 in each screen) and in vitro screens were performed 3 times (coverage > 160 x on day 7 in each screen) independently.

**Library construction for deep sequencing**

To quantify the enrichment of sgRNA, genomic DNA was extracted by TIANamp Genomic DNA Kit (TIANGEN BIOTECH) according to manufacturer’s protocol. The sgRNA sequence was PCR-amplified using high-fidelity polymerase (NEB) with barcoded primers from genomic DNA for library construction, followed by deep sequencing (Illumina). The raw data of deep sequencing was trimmed to leave behind only sgRNA sequence with ENCoRE software (Trümbach et al., 2017). After comparison to the reference sgRNA
sequence, the reads of each individual sgRNA in each sample were normalized within each sample as reads per million reads to offset the differences of sequencing depth among samples. For each gene, a P value was calculated by paired Student’s t test for examining the differences of gRNA abundance between input and end point for four gRNAs.

Flow cytometry
Single-cell suspension was prepared from blood, lymph nodes, spleens or tumors. Cell surface proteins were stained with indicated antibodies in the presence of Fc receptor block in FACS buffer (PBS containing 1% FBS, 2 mM EDTA, 100 units/ml penicillin and 100 µg/ml streptomycin) at 4°C for 15 min. Intracellular staining for intracytoplasmic and intranuclear proteins were performed with Transcription Factor Staining Buffer kit according to manufacturer’s instructions (BD Pharmingen). Dead cells were excluded by DAPI (BioLegend) staining or LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen). EdU labeling was performed with Click-iT EdU Flow Cytometry Assay Kits (Invitrogen) according to manufacturer’s protocol. Annexin V labeling was performed with Annexin V-FITC Apoptosis Detection Kit (Invitrogen) according to manufacturer’s instructions. Antibodies for staining were from BD Pharmingen, BioLegend, or Invitrogen as follows: anti-Thy1.1 (OX-7), anti-CD8α (53-6.7), anti-CD45.2 (104), anti-CD45.1 (A20), anti-ICOS (C398.4A), anti-CD25 (PC61), anti-PD-1 (29F.1A12), anti-TIM-3 (RMT3-23), anti-LAG-3 (CSB7W), anti-IFNy (XMG1.2), anti-TNFα (MP6-XT22), anti-GZMB (GB11), anti-Ki-67 (SolA15) and anti-IRF4 (IFR4.3E4). Streptavidin-conjugated fluorochrome were from BioLegend or Invitrogen. Samples were analyzed by a LSRFortessa cytometer (BD).

Western blot
Cas9;OT-I cells (11 million) transduced with indicated sgRNA were collected and lysed with lysis buffer. The soluble fractions of cell lysates were isolated by centrifugation at 20,000 g at 4°C for 10 min. Proteins were denatured with the addition of 6 x SDS sampling buffer and incubated at 95°C for 5 min. Protein samples were subjected to SDS-PAGE and immunoblotting analysis.

RNA sequencing and RT-qPCR
Cas9;OT-I cells (6 million) expressing indicated sgRNAs were injected into wild-type mice via tail vein. At 7 days post-activation, Thy1.1+ cells from spleen were sorted using a S3e cell sorter. Total RNA was extracted with RNAprep pure Cell Kit (TIANGEN BIOTECH) according to manufacturer’s protocol. Library construction and deep sequencing were performed by BGI (Beijing). For validation of selected genes by quantitative PCR (qPCR), total RNA of Thy1.1+ cells expressing indicated sgRNAs was prepared as described above. RNA was reversely transcribed to cDNA using the TIANScript II RT Kit (TIANGEN BIOTECH) according to manufacturer’s instructions. qPCR was performed with indicated primers using Talent qPCR PreMix (SYBR Green) (TIANGEN BIOTECH) under cycling condition: 95°C for 3 min followed by 40 cycles of (95°C for 10 s, 60°C for 30 s) using CFX96 Real-Time PCR Detection System (Bio-Rad). mRNA expression was normalized to β-Actin. qPCR Primers used in this study were as follow:

| Primer ID   | Primer Sequence                       |
|-------------|---------------------------------------|
| Pola1-F     | AAGAGTTACTTCAGAGGGTGAGCGA; Pola1-R    | TTGTCTTTCGCTCTACCACG  |
| Prim1-F     | TCAGCAAGGGGAGTCAATCCG; Prim1-R        | TTGAAGAATCGGCTCCAGG |
| Prim2-F     | TGAGCTATGTAAGGAACGCG; Prim2-R         | TTGAAGGCAAATCCAGGAC |
| Dna2-F      | TCCGAGAAGTTAGGCATTTCG; Dna2-R         | TTGAAGGAAATCAGCAGA |
| Mcm2-F      | TTCCCGCTTTATGGTGTCCTG; Mcm2-R         | ACCATTAGCACAACCTTCCTCC |
| Cdc45-F     | TCGAAGAGTATGTGAAACCGG; Cdc45-R        | TTATGACGGTAAAGTGGGAG |
| Orc1-F      | CCCTAAACTCCTACACCTTATCG; Orc1-R       | CAGAAACACTGAAACCTCAG |
| Rfl1-F      | AAACCAACTACCTCAGGATTGC; Rfl1-R        | TTCTGATGGAGGGTGTC |

mRNA stability assay
Thy1.1+ cells expressing indicated sgRNAs were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 µM ionomycin for 4 hours, followed by actinomycin D (Sigma) treatment for 0, 2 and 4 hours. mRNA levels were detected by RT-qPCR normalized to β-Actin.

QUANTIFICATION AND STATISTICAL ANALYSIS

The statistical information of each experiment, including the statistical methods, the P value and sample numbers (n) were shown in figure legends. GraphPad Prism 8 was used to plot all graphs and to perform statistical and quantitative assessments. Error bars represent standard error of mean (SEM).