Tumor suppressor BAP1 is essential for thymic development and proliferative responses of T lymphocytes

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Loss of function of the nuclear deubiquitinating enzyme BRCA1-associated protein-1 (BAP1) is associated with a wide spectrum of cancers. We report that tamoxifen-induced BAP1 deletion in adult mice resulted in severe thymic atrophy. BAP1 was critical for T cell development at several stages. In the thymus, BAP1 was required for progression through the pre–T cell receptor checkpoint. Peripheral T cells lacking BAP1 demonstrated a defect in homeostatic and antigen-driven expansion. Deletion of BAP1 resulted in suppression of E2F target genes and defects in cell cycle progression, which was dependent on the catalytic activity of BAP1, but did not require its interaction with host cell factor-1 (HCF-1). Loss of BAP1 led to increased monoubiquitination of histone H2A at Lys119 (H2A119ub) throughout the T cell lineage, in particular in immature thymocytes, but did not alter trimethylation of histone H3 at Lys27 (H3K27me3). Deletion of BAP1 also abrogated B cell development in the bone marrow. Our findings uncover a nonredundant function for BAP1 in maintaining the lymphoid lineage.

INTRODUCTION

BRCA1-associated protein-1 (BAP1) is a member of the ubiquitin C-terminal hydrolase (UCH) subfamily of deubiquitinating enzymes (DUBs) that has been studied mostly for its tumor suppressor function. Loss-of-function mutations of BAP1 have been identified in a variety of solid tumor types, including malignant pleural mesothelioma, uveal melanoma, clear cell renal carcinoma, intrahepatic cholangiocarcinoma, lung, and breast, and have been strongly linked to metastasis and poor prognosis (1).

BAP1 is ubiquitously expressed, and its constitutive deletion is embryonic lethal in mice (2). We and others recently reported that induced systemic deletion of BAP1 in adult mice leads to myelodysplastic syndrome with splenomegaly, leukocytosis, and myeloid progenitor expansion. The mice exhibit monocytosis and neutrophilia, as well as thrombocytopenia and anemia, reminiscent of chronic myelomonocytic leukemia in humans (2, 3). A physiological role for BAP1 in the immune system is unknown.

How BAP1 functions at the molecular level is also incompletely understood but appears to be context-dependent (1). BAP1 binds and modulates the E3 ubiquitin ligase activity of the BRCA1/BARD1 complex, a critical regulator of the DNA damage response (4, 5). In this context, inhibition of BAP1 was shown to sensitize cells to radiation, resulting in an impaired DNA damage response and S-phase retardation (5). On the other hand, ectopic expression in BAP1-negative H226 mesothelioma cells accelerated G1-S progression, leading to increased cell death. However, this process was found to be BRCA1-independent (6). These findings support a common function for BAP1 as a cell cycle regulator. BAP1 was found to associate with host cell factor-1 (HCF-1) (2, 7–9). Unable to bind DNA directly, HCF-1 recruits histone methyltransferases to the transcription factor E2F1, allowing the expression of E2F1 target genes that control the cell cycle (10, 11). It has been proposed that BAP1 recruits HCF-1 and enhances its activity by deubiquitinating HCF-1 itself or HCF-1–associated proteins, such as O-linked β-N-acetylglucosaminetransferase (OGT), thus promoting cell cycle progression and proliferation (2, 8, 12). BAP1 and its Drosophila homolog Calypso are members of the polycomb group of chromatin modifiers. Polycomb repressor complex 1 (PRC1) silences genes expression though chromatin compaction and H2AK119ub, a histone modification involved in the regulation of transcription initiation and elongation and DNA repair (13, 14). BAP1, in complex with additional sex combs-like1 or -2 (ASXL1/2), deubiquitinates H2AK119 through a process conserved from fly to human (15–17) and thus limits the repressive function of PRC1. More recently, BAP1 has been suggested to regulate polycomb repressor complex 2 (PRC2) activity and thereby trimethylation of histone 3 at Lys27 (H3K27me3), another epigenetic process involved in gene silencing (3).

Here, we report that BAP1 deletion in adult mice resulted in severe thymic atrophy and complete loss of the T cell lineage. B cell development in the bone marrow was also abrogated, suggesting a broader, nonredundant function for BAP1 in maintaining the lymphoid lineage. In thymocytes, BAP1 deficiency resulted in a block at the double-negative 3 (DN3) stage before the pre–T cell receptor (pre-TCR) checkpoint. Peripheral T cells exhibited a defect in homeostatic and antigen-driven expansion, and the numbers of peripheral T cells were also decreased in CD4–Cre–driven T cell–conditional BAP1 knockout (KO) mice. BAP1 deficiency resulted in suppression of E2F target genes and defects in cell cycle progression. Although the deubiquitinating activity...
of BAP1 was required for its function in T cells and correlated with increased H2AK11ub levels, its ability to interact with HCF-1 was dispensable. Our observations define a nonredundant role for BAP1 in the maintenance and function of the T cell lineage.

RESULTS
Deletion of BAP1 resulted in severe thymic atrophy and loss of DN thymocytes
We previously generated Bap1fl/fl Rosa26CreERT2 mice that express a tamoxifen-inducible Cre recombinase ubiquitously from the Rosa26 locus to induce systemic deletion of BAP1 in adult mice (2). Tamoxifen-treated Bap1fl/fl Rosa26CreERT2 mice displayed severe thymic atrophy and nearly complete loss of cellularity after 2 weeks, a phenotype that was never observed in tamoxifen-treated Bap1wt/wt Rosa26CreERT2 control mice (Fig. 1A). Histopathology revealed thymic atrophy in both the cortex and the medulla of the thymus (Fig. 1B). As early as 1 week after tamoxifen treatment, thymocyte numbers were decreased in these mice (Fig. 1C).

Thymocyte development progresses through sequential stages defined by the expression of CD4, CD8, c-Kit, CD25, and CD44 (for full set of markers used to identify all hematopoietic populations, see table S2). Enumeration of these individual thymocyte subsets revealed a decrease in all immature populations—including early thymic progenitors (ETPs), all DN cells, immature single-positive (ISP) cells, and double-positive (DP) cells—in Bap1fl/fl Rosa26CreERT2 mice compared with Bap1wt/wt Rosa26CreERT2 mice 1 week after tamoxifen treatment (Fig. 1D). The numbers of mature-stage CD4 and CD8 single-positive (SP) cells were not as reduced (Fig. 1D). We also observed strongly reduced numbers of γδ T cells in the thymus 1 week after BAP1 deletion. γδ T cells separate from the αβ T cell lineage at the DN2 stage (18). In contrast, γδ T cells in the spleen were normal in tamoxifen-treated Bap1fl/fl Rosa26CreERT2 mice (fig. S1A). Numbers of thymic natural killer cells, which populate the thymus after originating from the bone marrow, were unchanged (fig. S1B).

To test whether the thymocyte defect was intrinsic to the hematopoietic lineage or due to BAP1 loss in the thymic stroma, we generated chimeric mice by transferring bone marrow from either CD45.2+ Bap1fl/fl Rosa26CreERT2 or Bap1wt/wt Rosa26CreERT2 mice into lethally irradiated congenic CD45.1+ C57BL/6 recipient mice. After allowing 5 weeks for reconstitution, we induced BAP1 deletion by tamoxifen injection (fig. S2A). Mice that had received Bap1fl/fl Rosa26CreERT2 bone marrow showed thymic atrophy (fig. S2B), with loss of all thymocyte subsets (fig. S2C). Collectively, these data suggested that BAP1 was intrinsically required for immature DN thymocyte development, whereas the loss of SP thymocytes was likely a consequence of reduced thymic output.

BAP1 deficiency resulted in expansion of hematopoietic progenitors in the bone marrow and myeloid cells but abrogated B cell development
Consistent with previous reports (2, 3), BAP1 deletion resulted in an expansion of hematopoietic stem cells (HSCs), common myeloid progenitors (CMPs), and granulocyte–monocyte progenitors (GMPs) as early as 7 days after tamoxifen injection (Fig. 2A). We also observed increases in numbers of peripheral neutrophils and monocytes (Fig. 2B). Whereas lymphoid-primed multipotent progenitors (LMPPs), the earliest cells of the lymphoid lineage, were similarly increased in tamoxifen-treated Bap1fl/fl Rosa26CreERT2 mice, common lymphoid progenitors (CLPs) were normal in numbers compared with Bap1wt/wt Rosa26CreERT2 mice (Fig. 2C). The observation that the expansion seen in HSCs and LMPPs was not reflected in increased CLPs may point to a defect in proliferation or survival at the CLP stage in the lymphoid lineage. Consistent with this notion, B cell development was impaired in tamoxifen-treated Bap1fl/fl Rosa26CreERT2 mice. B cell precursors progress through the pre–pro-B, pro-B, pre-B, and immature B cell stages before becoming mature B cells (19). Most of these developmental stages were strongly impaired 1 week after BAP1 deletion (Fig. 2D). These findings suggested a broad requirement for BAP1 in the development and/or maintenance of the lymphoid lineage and highlight the dichotomy between BAP1 function in lymphoid and myeloid cells.

BAP1 was required for progression through the DN3 stage of thymocyte development
We further investigated the impact BAP1 loss had on the T cell lineage. To circumvent the rapid loss of immature thymic precursors upon BAP1 deletion in vivo, we cultured lineage-depleted bone marrow cells from Bap1fl/fl Rosa26CreERT2 or Bap1wt/wt Rosa26CreERT2 mice on OP9-DL1 cells in vitro, deleted BAP1 by adding 4-hydroxytamoxifen (4-OHT) on day 2 of

Fig. 1. BAP1 deficiency results in thymic atrophy and loss of thymocyte populations. (A and B) Thymi (A) and hematoxylin and eosin staining of thym (B) from Bap1fl/fl Rosa26CreERT2 and Bap1wt/wt Rosa26CreERT2 mice 2.5 weeks after tamoxifen treatment. (C) Thymocyte numbers in Bap1fl/fl Rosa26CreERT2 and Bap1wt/wt Rosa26CreERT2 mice 2.5 weeks after tamoxifen treatment. (D) Cell numbers of the indicated thymocyte populations in Bap1fl/fl Rosa26CreERT2 and Bap1wt/wt Rosa26CreERT2 mice 1 week after tamoxifen treatment, as determined by flow cytometry analysis. All data are representative of at least three independent experiments. For (C) and (D), each symbol represents an individual mouse and the line shows the median. *P < 0.05, **P < 0.01, ****P < 0.0001 based on unpaired Student’s t test.

culture, and analyzed cells by flow cytometry at different time points. Wild-type (WT) cells progressed through the stages of early T cell development, with the majority of cells being closer to the DN2 stage by day 6 and cells transitioning to the DN3 stage by day 9 and to the DP stage by day 12 (Fig. 3, A and B). In contrast, BAP1-deficient cells progressed normally through the DN1, DN2, and DN3 stages but exhibited a block at the DN3 stage. In the absence of BAP1, cells were unable to transition to the DP stage (Fig. 3, A and B). At the end of the culture at day 15, the majority of WT cells were at the DP stage, whereas BAP1-deficient cells remained at the DN3 stage (Fig. 3B). We also observed impaired expansion of BAP1-deficient cells in OP9-DL1 cultures (Fig. 3C).

Given the drop in numbers of ETP and DN2 cells observed in vivo (Fig. 1D), it was likely that BAP1 also regulated an earlier checkpoint that is not captured in our in vitro studies because of the time it took to fully delete BAP1 after 4-OHT addition (about 48 hours). Even so, our data suggested that BAP1 was critically required for thymocytes to progress beyond the DN3 stage.

To further define the function of BAP1, we performed RNA sequencing (RNA-seq) on sorted tamoxifen-treated Bap1<sup>fl/fl</sup>Rosa26<sup>CreERT2</sup> and Bap1<sup>fl/fl</sup>Rosa26<sup>CreERT2</sup> DN3 cells from OP9-DL1 cultures on day 14. Initial analysis revealed that a large number of genes were differentially expressed in BAP1-deficient DN3 cells, roughly evenly split between genes significantly up- and down-regulated at least twofold (Fig. 3D). We focused our analysis on a set of genes that are dynamically regulated during the transition from the DN3 to DN4 stage of thymocyte development (20). We observed that genes that are down-regulated during the normal transition from the DN3 to DN4 stage were more highly expressed in BAP1-deficient DN3 cells compared with WT controls, whereas those genes that are up-regulated during the same transition were expressed at lower levels in BAP1-deficient DN3 cells (Fig. 3, E and F). This suggested that BAP1-deficient DN3 cells were developmentally immature in comparison with WT DN3 cells. We performed gene set enrichment analysis (GSEA) against the hallmark gene sets, a refined curation of gene sets from the Molecular Signatures Database (MSigDB) (21). We found a highly significant and uniform down-regulation of E2F target genes in BAP1-deficient DN3 cells (Fig. 3, E and F), suggesting deregulation of the cell cycle. Similarly, gene sets representing G2-M checkpoint and MYC target genes were significantly down-regulated in the absence of BAP1 (Fig. 3E).

Generally, these findings highlighted a function of BAP1 as a cell cycle regulator in thymocytes. Defects in cell cycle progression in BAP1-deficient DN3 cells were likely to contribute to the observed block at this stage because the transition to DN4 and ISP after passage through the pre-TCR checkpoint is accompanied by massive cell expansion (22, 23).

**BAP1 deubiquitinating activity, but not HCF-1 binding, was required for thymocyte development**

We next wanted to gain further mechanistic insight into how BAP1 regulated thymic T cell development. In several tissues, BAP1 has been shown to reside within a larger protein complex, termed the BAP1 core complex (2, 7–9). Coimmunoprecipitation confirmed robust interactions with known complex members HCF-1, OGT, ASXL1, and KDM1B in thymocytes (Fig. 4A). In contrast to a previous report that BAP1 interacts with EZH2 in myeloid cells (3), BAP1 did not interact with EZH2 in thymocytes (Fig. 4A).

We had strong evidence of repressed E2F target genes in BAP1-deficient DN3 thymocytes by RNA-seq. BAP1 has been suggested to recruit and modify the activity of HCF-1, a known regulator of E2F target genes (2, 8, 12). Members of the BAP1 core complex, including HCF-1 and OGT, have been described as BAP1 substrates in other systems (2, 8, 12). Western blot analysis revealed marginally reduced protein levels of HCF-1 but not OGT in BAP1-deficient DN3 cells, suggesting that BAP1 did not regulate HCF-1 stability in thymocytes (Fig. 4B).

To probe the involvement of HCF-1, we reconstituted Bap1<sup>KO/KO</sup>Rosa26<sup>CreERT2</sup> and Bap1<sup>KO/KO</sup>Rosa26<sup>CreERT2</sup> lineage-negative bone marrow progenitors with either WT or a mutant form of BAP1 that is selectively deficient in its ability to interact with HCF-1 (4A, NHNY to AAAA) (8). Both WT and 4A mutant BAP1 were able to rescue T cell differentiation and DP cell generation in the OP9-DL1 system upon BAP1 deletion (Fig. 4, C and D), demonstrating that the ability to recruit HCF-1 was dispensable for BAP1 function. This suggested that BAP1 regulated E2F target gene expression independently of HCF-1.

Next, to determine whether the deubiquitinating activity of BAP1 was required for T cell differentiation, Bap1<sup>fl/fl</sup>Rosa26<sup>CreERT2</sup> lineage-negative bone marrow progenitors were reconstituted with WT or a catalytically dead mutant BAP1 (C91A) and cultured in the OP9-DL1 system. In clear contrast to WT and mutant BAP1 (4A), mutant BAP1 (C91A) was not able to restore T cell development (Fig. 4, C and D), demonstrating the requirement for BAP1 deubiquitinating activity.
suggested that the deubiquitinating activity of BAP1 was required for T cell differentiation in this system.

Deubiquitination of H2AK119 is the primary function of the BAP1 homolog Calypso in Drosophila and is evolutionarily conserved to human (15–17, 24). We performed flow cytometric analysis of H2AK119ub levels comparing WT and BAP1-deficient DN3 cells generated in the OP9-DL1 system. H2AK119ub was strongly enhanced in the absence of BAP1 (Fig. 4E). A previous report had found increases in H3K27me3 and EZH2 expression in BAP1-deficient myeloid progenitors (3). However, we did not detect differences in H3K27me3 levels or in expression of EZH2 or other members of the PRC1 and PRC2 complexes (Fig. 4, E and F, and fig. S3). Notably, EZH1 RNA levels were increased in BAP1-deficient DN3 cells but did not translate into changes in H3K27me3 (fig. S3). We also performed mass spectrometric analysis of a wide array of histone modifications comparing WT and BAP1-deficient DN3 cells isolated from OP9-DL1 cocultures (Fig. 4F). We found a decrease in monomethylation of H4K20, which has previously been observed in BAP1-deficient myeloid progenitors (3). This decrease correlated with an increase in tri-methylation of H3S10 in BAP1-deficient cells (Fig. 4F). Phosphorylation of H3S10 in BAP1-deficient cells (Fig. 4F). The dynamic regulation of the H4K20 methylation state is associated with cell proliferation and required for normal cell cycle progression (25–27), thus further suggesting a proliferation defect in BAP1-deficient cells. We observed a decrease in phosphorylation of H3S10 in BAP1-deficient cells (Fig. 4F). Phosphorylation of H3S10, a hallmark of mitosis (28), normally correlates with global deubiquitination of H2AK119 during the G2-M transition. H2AK119 ubiquitination has been shown to interfere with H3S10 phosphorylation, in part by preventing access for Aurora B kinase.

Fig. 3. Loss of BAP1 blocks T cell differentiation at the DN3 stage in vitro. Bone marrow progenitor cells from Bap1<sup>fl/fl</sup>Rosa26<sup>CreERT2</sup> and Bap1<sup>WT/wt</sup>Rosa26<sup>CreERT2</sup> mice were cultured on OP9-DL1 stromal cells for the indicated number of days, with deletion of BAP1 induced at day 2 by 4-OHT addition (n = 3). (A) Staining profiles of GFP<sup>−</sup> were cultured on OP9-DL1 stromal cells for the indicated number of days, with deletion of BAP1 induced at day 2 by 4-OHT addition (n = 3). (A) Densities of logFC values of each gene in the indicated gene data set (orange) compared with BAP1 WT versus KO DN3 data sets with an FDR less than 20% are shown. (B) GSEA of BAP1 WT versus KO DN3 cell data sets. Gene set–level heat maps (left) and FDR (right) of gene sets that are differentially enriched in = 3). (A) Proportions within GFP<sup>−</sup> CD45<sup>+</sup> cells (B) and absolute numbers (C) of T cell populations at the indicated time points. (D) RNA-seq was performed on BAP1 WT and KO DN3 cells (CD45<sup>+</sup> lineage<sup>−</sup> CD4<sup>+</sup> CD8<sup>−</sup> CD44<sup>−</sup> c-Kit<sup>−</sup> CD25<sup>−</sup>) isolated from OP9-DL1 cocultures at day 14. A volcano plot of logFC (KO-WT) versus nominal P values (−log<sub>10</sub>) is shown. Differentially expressed genes (1.5-fold, FDR < 0.1) are highlighted in dark blue, and genes in the hallmark E2F target gene set are highlighted in orange. (E) GSEA of BAP1 WT versus KO DN3 cell data sets. Gene set–level heat maps (left) and FDR (right) of gene sets that are differentially enriched in BAP1 WT versus KO DN3 data sets with an FDR less than 20% are shown. (F) Densities of logFC values of each gene in the indicated gene data set (orange) compared with the logFC values of all genes (blue). Each green dot represents one gene in the indicated data set.

BAP1 was dynamically expressed and regulated H2AK119ub and not through the recruitment of HCF-1 to regulate cell cycle progression in T cells.

Analysis of H2AK119ub in bone marrow progenitor populations in tamoxifen-treated Bap1fl/flRosa26CreERT2 mice and tamoxifen treated Bap1fl/flRosa26CreERT2 mice. (Fig. 5B), consistent with the uniform expression of BAP1 in these cells. H2AK119ub was increased in BAP1-deficient DN thymocytes, which was again consistent with high BAP1 expression in these cells (Fig. 5B), suggesting that BAP1 had a nonredundant function of limiting ubiquitination of H2AK119 in early thymic development. The low abundance of ETP in tamoxifen-treated Bap1fl/flRosa26CreERT2 mice prevented us from measuring H2AK119ub levels in these cells. The relatively low expression of BAP1 in the bone marrow of WT mice and more modest, although statistically significant, increases of H2AK119ub in the absence of BAP1 suggested redundancy with other H2A deubiquitinases. Mature SP thymocytes from WT mice contained reduced H2AK119ub levels compared with more immature thymic populations, suggesting that this histone mark was not rapidly turned over in these cells (Fig. 5B).

Flow cytometric analysis of H2AK119ub revealed uniformly increased levels in all bone marrow progenitor populations in tamoxifen-treated Bap1fl/flRosa26CreERT2 mice compared with tamoxifen-treated Bap1fl/flRosa26CreERT2 mice (Fig. 5B), consistent with the uniform expression of BAP1 in these cells. H2AK119ub was increased in BAP1-deficient DN thymocytes, which was again consistent with high BAP1 expression in these cells (Fig. 5B), suggesting that BAP1 had a nonredundant function of limiting ubiquitination of H2AK119 in early thymic development. The low abundance of ETP in tamoxifen-treated Bap1fl/flRosa26CreERT2 mice prevented us from measuring H2AK119ub levels in these cells. The relatively low expression of BAP1 in the bone marrow of WT mice and more modest, although statistically significant, increases of H2AK119ub in the absence of BAP1 suggested redundancy with other H2A deubiquitinases. Mature SP thymocytes from WT mice contained reduced H2AK119ub levels compared with more immature thymic populations, suggesting that this histone mark was not rapidly turned over in these cells (Fig. 5B).

Using this approach, we did not observe increased H3K27me3 in any bone marrow progenitor or thymocyte population, including CMPs.
Tamoxifen-induced deletion of BAP1 also resulted in a moderate reduction in peripheral T cell numbers. Bap1 T cells (fig. S5B), which suggested a role for BAP1 in peripheral T cell populations, including naïve, memory, and effector subsets, but not in CD8+ T cells (fig. S5A). Although they also showed enhanced H2AK119ub levels, we did not observe enhanced H3K27me3 levels in these cells. On the contrary, all three splenic myeloid populations showed a slight but statistically significant reduction in H3K27me3 (fig. S4B). We also extended our analysis of BAP1 expression and corresponding histone modifications into B cell progenitors in the bone marrow. BAP1 was expressed at lower levels compared with DN thymocytes (fig. S4C). However, similar to these immature T cell populations, B cell progenitors showed increased H2AK119ub levels, further supporting a nonredundant role for BAP1 in lymphopoiesis. Together with decreasing BAP1 expression, differences in H2AK119ub levels also decreased as B cells differentiated (fig. S4D). Our flow cytometric analysis revealed a critical role for BAP1 in regulating H2AK119ub monoubiquitination throughout the hematopoietic system, but most prominently in immature thymocytes, which also exhibited the highest BAP1 expression.

BAP1 was continuously required to maintain peripheral CD4+ and CD8+ T cells

Tamoxifen-induced deletion of BAP1 also resulted in a moderate reduction in peripheral CD4+ T cells and a loss in CD8+ T cells in the spleen (fig. S5A). The reduction in cell numbers was more pronounced in central memory and effector memory as compared with naïve T cells (fig. S5B), which suggested a role for BAP1 in peripheral T cell maintenance. However, it was unclear to what extent the reduced thymic output in tamoxifen-treated Bap1fl/fl Rosa26CreERT2 mice contributed to the decrease in peripheral T cell numbers.

To directly assess a potential role for BAP1 in peripheral T cells, we generated Bap1fl/fl Cd4-Cre+ mice, which delete BAP1 beginning with CD4 expression at the DP stage in the thymus. As expected, immature thymocyte subsets from ETP to ISP were normal in numbers in 8-week-old Bap1fl/fl Cd4-Cre+ mice as compared with WT controls (fig. S6A). Whereas DP cell numbers were also normal in 8-week-old Bap1fl/fl Cd4-Cre+ mice, mature CD4+ T cells were slightly and mature CD8+ T cells were more strongly decreased (fig. S6A). Consistent with BAP1 deletion at about the DP stage, we observed moderately increased levels of H2AK119ub in DP as well as CD4+ SP and CD8+ SP T cells (fig. S6B). Given that we did not observe enhanced H2AK119ub in SP thymocytes after acute tamoxifen-induced BAP1 deletion, this suggested that histone modifications may accumulate over time in BAP1-deficient cells in Bap1fl/fl Cd4-Cre+ mice. CD8 SP thymocytes from these mice showed a moderate increase in H3K27me3 (fig. S6C).

Numbers of peripheral CD4+ and CD8+ T cells were significantly reduced in spleens of 8-week-old Bap1fl/fl Cd4-Cre+ mice (fig. 6A). Whereas both naïve and memory peripheral T cells were affected, the loss in naïve T cell populations was more pronounced (Fig. 6B). Both CD4+ and CD8+ peripheral T cells were further reduced in 10-month-old Bap1fl/fl Cd4-Cre+ mice (fig. S7A), suggesting that these cells were gradually lost over time. Again, the loss in naïve peripheral T cells in aged Bap1fl/fl Cd4-Cre+ mice was more pronounced compared with the memory population (fig. S7B), whereas we had observed a stronger impact on the memory population after acute tamoxifen-induced BAP1 deletion in peripheral T cells. Thymus, mesenteric lymph node, and spleen of aged Bap1fl/fl Cd4-Cre+ mice showed normal architecture and no signs of pathology (fig. S7C).

BAP1 was expressed in peripheral naïve, memory, and effector T cell populations at levels similar to mature SP thymocytes, but lower than in DN thymocytes (Fig. 6C). Global H2AK119ub and H3K27me3 levels were lower in peripheral T cells compared with immature thymocytes (Fig. 6, D and E). Consistent with a continued role for BAP1 in peripheral T cells, acute tamoxifen-induced BAP1 deletion in Bap1fl/fl Rosa26CreERT2 mice within 1 week resulted in moderate increases in H2AK119ub in peripheral T cells, mainly in effector and memory cells (Fig. 6D). H2AK119ub levels were much more strongly increased in peripheral T cells, including the naïve population, from 8-week-old Bap1fl/fl Cd4-Cre+ mice (fig. 6E), suggesting an accumulation with prolonged BAP1 deficiency. Acute tamoxifen-induced BAP1 deletion in Bap1fl/fl Rosa26CreERT2 mice led to moderate decreases in H3K27me3 in most peripheral T cell populations (Fig. 6E). In contrast, naïve CD4 and CD8 T cells, but not memory or effector subsets, in Bap1fl/fl Cd4-Cre+ mice exhibited slightly increased H3K27me3 (Fig. 6F) and also expressed more EZH2 (Fig. 6F). The fact that we observed increases in H3K27me3 only in Bap1fl/fl Cd4-Cre+ mice may
suggest that it was a secondary, presumably compensatory, mechanism rather than an immediate consequence of BAP1 loss. Collectively, these data demonstrated that the function of BAP1 in the T cell lineage extended beyond the thymus and that BAP1 was essential to maintain the peripheral T cell pool.

**BAP1 deficiency resulted in impaired antigen-driven proliferation and effector response in peripheral T cells**

We next investigated the capacity of BAP1-deficient cells to homeostatically proliferate. To this end, we transferred isolated naïve CD4 T cells from tamoxifen-treated Bap1fl/flRosa26CreERT2 mice or WT controls into Rag2−/− mice, either separately or mixed at a 1:1 ratio. After 3 weeks, we analyzed splenic CD4+ T cells. Irrespective of whether they had been transferred alone or together with control T cells, BAP1-deficient CD4+ T cells were not able to reconstitute the CD4 compartment (Fig. 7A), demonstrating a defect in homeostatic proliferation.

When naïve CD4 T cells from tamoxifen-treated Bap1fl/flRosa26CreERT2 mice or WT controls were cultured with anti-CD3/anti-CD28 for 48 hours (Fig. S10A), again, initial T cell proliferation and effector response in peripheral T cells by RNA-seq data demonstrating that BAP1-deficient T H 0, T H 1, and T H 17 clustered closely with their WT counterpart in principal components analysis (fig. S8C). A large proportion of genes deregulated between WT and BAP1-deficient T cells was shared between more than one polarized subset (fig. S8D). GSEA revealed a down-regulation of E2F target genes (Fig. 7B and fig. S8E), consistent with our results from BAP1-deficient DN3 thymocytes. Accordingly, Rag2−/− recipient mice that had been reconstituted with Bap1fl/flRosa26CreERT2 cells before BAP1 deletion by tamoxifen treatment were completely protected from T H 17-mediated experimental autoimmune encephalomyelitis (EAE) (fig. S9), further demonstrating the inability of BAP1-deficient T cells for antigen-driven expansion.

A similar deregulation of E2F target genes was also observed in naïve T cells from Bap1fl/flCd4-Cre− mice that had been stimulated with anti-CD3/anti-CD28 for 48 hours (fig. S10A). Again, initial T cell activation was not impaired in Bap1fl/flCd4-Cre− T cells, as evidenced by comparable up-regulation of early activation markers CD69 and
CD25 (fig. S10B). Cell recovery after 6 days of culture was strongly reduced in BAP1-deficient T cells (Fig. 7C). This defect could not be rescued by signals from cocultured WT cells (fig. S10C) or the addition of IL-2 or IL-7 (fig. S10D).

Cell cycle analysis through 5-bromo-2′-deoxyuridine (BrdU) incorporation showed a pronounced delay and partial block in the G0-G1- to S-phase transition upon anti-CD3/anti-CD28 stimulation in BAP1-deficient CD4 T cells compared with WT controls (Fig. 7D). Again, we found BAP1 to interact with the BAP1 core complex members in peripheral CD4 T cells (fig. S11A). HCF-1 and OGT levels were slightly reduced in BAP1-deficient CD4 T cells (fig. S11B), and BAP1 catalytic activity, but not its interaction with HCF-1, was required to maintain peripheral T cell proliferation (fig. S11C). Profiling of histone post-translational modifications by mass spectrometry of WT and BAP1-deficient CD4 T cells after 48 hours of anti-CD3/anti-CD28 stimulation largely revealed the same pattern that we had observed for DN3 thymocytes, with reduced monomethylation of H4K20, increased trimethylation of H4K20, and reduced phosphorylation of H3S10 (fig. S11D).

These findings confirmed the role for BAP1 in cell cycle regulation in peripheral T cells. In contrast to the block in thymic differentiation, however, cell cycle progression in peripheral T cells was delayed but not strictly blocked in naive T cells from Bap1fl/flRosa26-Cre+ mice (Fig. 7E). A detailed time-course analysis of proliferation and cell expansion in CD4 T cell cultures over the course of 4 days revealed that the initial defect in proliferation was overcome by a small subset of BAP1-deficient T cells that showed normal expansion afterward (Fig. 7E).

Collectively, our data demonstrated that BAP1 was required in peripheral T cells for homeostatic and antigen-driven expansion. Lack of BAP1 did not interfere with initial T cell activation but restricted cell cycle progression and expansion, consistent with its general function in the T cell lineage.

DISCUSSION

Here, we report that BAP1 was essential within the hematopoietic system to maintain the T lymphocyte lineage, both during early T cell development in the thymus and later for mature T cell function in the periphery. Throughout the life span of T cells, BAP1 was essential to facilitate cell cycle progression during critical proliferative steps.
responses, either during pre-TCR and TCR selection of thymocytes or homeostatic and antigen-driven expansion of peripheral T cells, explaining the severe thymic atrophy and reduced peripheral T cell numbers observed in tamoxifen-treated Bap1floxedRosa26CreERT2 and Bap1floxedCd4-Cre mice. We confirmed that the deubiquitinase activity was essential for BAP1 function in T cells. BAP1-deficient thymocytes or peripheral T cells reconstituted with WT BAP1, but not a catalytically inactive C91A mutant BAP1, proliferated and differentiated normally.

Upon tamoxifen-induced deletion of BAP1, we observed enhanced global H2AK119 monoubiquitination in T lymphocyte populations and throughout the hematopoietic system in the bone marrow, thymus, and secondary immune organs, consistent with the evolutionarily conserved function of BAP1 as a H2AK119 deubiquitinase (15–17). Although H2AK119ub is well known to be linked to the regulation of cell proliferation, the precise mechanism, whether primarily transcriptional or epigenetic, is insufficiently understood. In proliferating cells, ubiquitinated H2AK119 is present throughout the cell cycle, but it is down-regulated during the G2-M transition and is not present on condensed chromosomes (28). Global deubiquitination of H2AK119 during the G2-M transition correlates with phosphorylation of H3S10, a hallmark of mitosis (28). H2AK119 ubiquitination has been shown to interfere with H3S10 phosphorylation, in part by preventing Aurora B kinase from accessing its histone substrate (28). We observed strongly reduced global H3S10 phosphorylation in BAP1-deficient thymocytes and activated peripheral T cells by mass spectrometry. The particularly high expression of BAP1 in thymocytes and the concomitant increase in global H2AK119ub in BAP1-deficient thymocytes likely support a direct role for BAP1 in the epigenetic control of cell cycle progression in these cells. In contrast, the more moderate increases in H2AK119ub seen in other cell types, including bone marrow progenitors and myeloid cells, might point to a more targeted transcriptional mechanism by which BAP1 regulates the expression of a set of target genes. This may explain the disparate effects of BAP1 loss on proliferation in those cells. A repression of HoxA genes in a PR2/EZH2-dependent manner has been suggested to account for the phenotype in those cells (3). One week after tamoxifen treatment, BAP1 deletion did not enhance H3K27me3 in the T cell lineage. The PR1 complex has been reported to facilitate bone marrow progenitor self-renewal and lineage specification by silencing inhibitory cell cycle regulators, including p16INK4A and p19ARF, through H2A ubiquitination (29–31). It appears likely that BAP1 functions to antagonize this pathway.

However, transcriptional mechanisms may also contribute to the impaired cell cycle progression in BAP1-deficient T cells. Transcriptional profiling revealed down-regulation of E2F target genes in both thymocytes and peripheral T cells in the absence of BAP1. Genes regulated through E2F family transcription factors are essential for cell cycle progression (10, 11). Previous reports have demonstrated the regulation of E2F target genes by BAP1 through its recruitment of the transcription factor HCF-1 (2, 8, 12), itself a component of the BAP1 core complex. HCF-1, which does not bind DNA directly, co-regulates gene expression through E2F interaction (10, 11). Although our studies demonstrate a down-regulation of E2F target genes in BAP1-deficient T cells, we found that BAP1 function did not depend on its ability to interact with HCF-1, which leaves the possibility that BAP1 interacts with E2F directly, as has been suggested previously (12), or that the deregulation of E2F genes is an indirect consequence of BAP1 action at the epigenetic level.

Our characterization of global H2AK119ub levels in BAP1-deficient immune cells by flow cytometry cannot address how changes in H2AK119 ubiquitination affect the epigenetic and/or transcriptional regulation of gene expression in a cell type–specific manner. This will be an important question for future studies. In particular, the molecular basis for the striking disparity in BAP1-mediated control of proliferation in lymphoid and myeloid cells warrants further investigation. Our findings in B progenitors in the bone marrow suggest that the earliest defect in the lymphoid lineage in BAP1-deficient mice resides at the level of common lymphoid precursors, leading to a reduced cellular input into both the B and T cell lineage, and also hint at additional B cell checkpoints controlled by BAP1 similar to the T cell lineage. However, our studies were not designed to fully elucidate the B cell phenotype in BAP1-deficient mice. Future research using conditional BAP1 deletion in the B cell lineage and culture systems optimized for B cell progenitors will help to address these questions. In conclusion, we have identified a nonredundant role for BAP1 in the T lymphocyte lineage, where it acts to ensure proper proliferative responses critical for T cell development and function.

MATERIALS AND METHODS

Study design

The goal of this study was to characterize the function of BAP1 in the T lymphocyte lineage using conditional BAP1 KO mice. All experimental studies were conducted under protocols approved by the Institutional Animal Care and Use Committee of Genentech Lab Animal Research in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Power calculations from past studies were used to calculate the number of mice needed to ensure statistical power. All animal studies were conducted on three to five biological replicates. No randomization or blinding was performed, but all results were confirmed by two or more independent experiments.

In vitro T cell differentiation

HSC-enriched bone marrow progenitor cells from Bap1fl/flRosa26CreERT2 and Bap1wt/wtRosa26CreERT2 mice were isolated using the Direct Lineage Cell Depletion Kit (Miltenyi Biotec). OP9-DL1 stromal cells were cocultured with these lineage-depleted bone marrow cells in the presence of Flt-3L (5 ng/ml) and IL-7 (5 ng/ml) for the first 7 days of culture. At day 7, Flt-3L (5 ng/ml) and IL-7 (5 ng/ml) were added to the cultures to induce deletion of Bap1. T cell differentiation was monitored at various time points by flow cytometry. OP9-DL1 stromal cells were excluded from analysis by a combination of forward and side scatter characteristics, green fluorescent protein (GFP) expression, and lack of CD45 expression.

Differential gene expression

Reads were mapped to the mouse mm10 genome using GSNAp (33). Differential expression analysis was performed using the voom/limma pipeline (34, 35). Briefly, we required genes to exhibit at least one count per million expression in at least three samples for downstream analysis. The filtered count matrix was then processed with voom before differential expression analysis using limma. Volcano plots were then drawn [logarithmic fold changes (logFCs) and nominal P values] using the standard testing functionality in limma. Differentially expressed genes were selected by identifying the genes with false discovery rate (FDR) less than 10% when tested against a minimal fold change of 1.5 using limma’s “treat” functionality (36).
**Gene set enrichment analysis**

GSEA was performed using CAMERA (37) with a prespecified intergene correlation value set to 0.02 (38) against the MSigDB hallmark gene sets (v5.1) (39) and a set of stage-dependent thymocyte development genes taken from Arenzana et al. (20). Gene sets at an FDR less than 20% were chosen for display in the gene set–level heat maps. To visualize the activity of a gene set per sample in the heat map, we generated single-sample gene set scores as previously described (40) and plot their row-wise $z$-transformed values.

**Histone purification and mass spectrometry**

Core histones were purified from frozen cell pellets by acid extraction, ion exchange column, and perchloric acid precipitation using the Histone Purification Mini Kit (Active Motif). Two micrograms of $\gamma\delta$-transformed values.

et al mark gene sets (v5.1) (38) GSEA was performed using CAMERA (37) with a prespecified in- SCIENCE IMMUNOLOGY  peripheral T cells.  Fig. S11. Deubiquitinating activity, but not HCF-1 binding, is required for BAP1 function in $\gamma\delta$-transformed values.


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Tumor suppressor BAP1 is essential for thymic development and proliferative responses of T lymphocytes


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Fueling T cell proliferation

Previous studies on BRCA1-associated protein-1 (BAP1) have documented its importance in suppressing development of myeloid leukemia. BAP1 is a deubiquitinase (DUB) that acts on histone H2A monoubiquitinated at Lys 119 (H2AK119ub), a chromatin modification associated with gene repression. Arenzana et al. report that BAP1 is essential for development of T cells in the thymus and for promoting peripheral T cell proliferation. The authors report that deletion of BAP1 impaired expression of genes associated with cell cycle progression in thymocytes and in peripheral T cells. In both cases, the effect of BAP1 deletion was dependent on the DUB activity of BAP1, calling for a closer examination of the role of H2AK119ub in T cell development and differentiation.