이학박사학위논문

SRG3에 의한 SWI/SNF 염색사 리모델링 복합체의 안정화 및 BAP1이 흉선세포 분화에 미치는 영향에 대한 연구

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지도교수 성노현

이 논문을 이학박사학위논문으로 제출함

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Submitted in partial fulfillment of the requirements

For the degree of

DOCTOR OF PHILOSOPHY

To the faculty of

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at

SEOUL NATIONAL UNIVERSITY

By

JUNG INKYUNG

Date Approved:

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ABSTRACT

T cells are major mediators of cell-mediated immune responses. T cell progenitors originating in the bone marrow enter the thymus, and then differentiate, undergo selection, and mature into functional T cells through a series of organized and complicated developmental stages. These multiple steps in T cell development are regulated by the transcriptional control of lineage-specific genes and by the sequential rearrangement of T cell receptor (TCR) genes. Switching the transcription of various genes, including Notch, GATA-3, E2A/HEB, Id proteins, c-Myb, ThPOK, Runx complex, and Ikaros family genes, on or off is critical for lineage commitment in T cells. In addition, the β-selection and positive selection, which depend on the successful rearrangement of TCR genes, are essential for the production of mature T cells. Chromatin status, which is regulated by histone modification enzymes and chromatin remodeling complex, ultimately determines whether gene expression is turned on or off.

Here, I studied the major components of the murine SWI/SNF complex. It was found that BRG1, SNF5, and BAF60a are targeted for ubiquitylation and degradation, but that SRG3 can protect them from degradation. Previous studies have shown that the SWI/SNF ATP-dependent chromatin remodeling complex is essential for the developmental transitions of thymocytes. In many studies, the stability of

the SWI/SNF complex has been shown to be regulated by ubiquitylation. In particular, SRG3 stabilizes SNF5, BRG1 and BAF60a by attenuating their proteasomal degradation, suggesting its essential role in stabilization of the SWI/SNF complex. It was also found that CHFR, an E3 ubiquitin ligase and known tumor suppressor that plays an essential role in cell cycle control and tumorigenesis, interacts with and ubiquitylates BRG1, SNF5, and BAF60a of the SWI/SNF complex, targeting them for degradation through a proteasome-mediated pathway. Moreover, It was found that SRG3 stabilizes these components by blocking their interaction with CHFR.

Second, I investigated the function of Bap1, a deubiquitinase involved in the regulation of cell growth and proliferation, in thymocyte development. Bap1-deficient mice showed developmental retardation in embryo and expansion of the myeloid lineage. In this study, to gain insight into the contributions of Bap1 to T cell development, the mouse *Bap1* gene was deleted cell specifically, using Lck-Cre-producing mouse strains. The total thymocyte number was significantly reduced in Bap1 conditional knockout mice. It was found that Bap1 plays a critical role in pre-TCR signaling and TCR-mediated T cell development and activation. Bap1-deficient T cells displayed the block from stage double negative (DN) 3 to DN4, and they exhibited reduced expression of TCRβ compared with controls. Thymic positive selection was also significantly decreased when Bap1 was knocked out in a TCR transgenic background.

TCR-mediated signaling events, including calcium flux and extracellular signal-regulated kinase (ERK) activation, were impaired in Bap1-deficient mice. Additionally, peripheral T cells were not efficiently activated upon TCR stimulation. Taken together, these findings suggest that Bap1 is a critical component of T cell development and that it participates in TCR signaling events required for thymocyte activation.

From these results, I conclude that: 1) the SWI/SNF chromatin remodeling complex for ordinary T cell development is stabilized by SRG3 through the blockade of CHFR activity, and 2) Bap1 expression in pre-TCR and TCR signal transduction is critical for successful thymocyte development and maturation.

Keywords:

T cell development, SWI/SNF complex, SRG3, CHFR, Bap1, ubiquitylation

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I.

INTRODUCTION

I-1. Thymocyte development and TCR signaling

The development of thymocytes from bone marrow-derived progenitor cells occurs within the thymus. It is characterized by the expression of two surface proteins, CD4 and CD8 coreceptors (Klein et al., 2014). The thymic lymphoid progenitor cells in the thymus lack expression of the CD4 and CD8, so they are referred to as double-negative (DN) thymocytes. The DN thymocytes have been further subdivided into four stages by the expression of two surface molecules CD44 and CD25, as follows: $CD44^{+}CD25^{-}$ (DN1), $CD44^{+}CD25^{+}$ (DN2), $CD44^{-}CD25^{+}$ (DN3) and $CD44^{-}CD25^{-}$ (DN4) (Ceredig and Rolink, 2002). The only DN3 thymocytes that have expressed a functional T cell receptor (TCR)-β chain on the surface with pre-Tα and CD3 element to form the pre-TCR mature to the $CD4^{+}CD8^{+}$ double-positive (DP) cells, otherwise the development is blocked at DN3 stage. This checkpoint is termed as β-selection (Shortman and Wu, 1996), which is a cascade of differentiation and proliferation events triggered specifically by pre-TCR signaling in precursors of TCRαβ T cells, after successful rearrangement of the TCRβ gene. Rearrangement is made possible by the expression of the linked RAG-1 and RAG-2 recombinase genes and by the accessibility of the TCR-coding loci in chromatin at the appropriate stages (Wolfer et al., 2002). Transcriptional induction of RAG genes and transcription factor-mediated opening of the TCR loci thus constitute aspects of T cell specifi-

cation.

The DP thymocytes are continued to mature by making a functional TCR-α chain and subsequently undergo the process of positive selection to further develop into either $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) thymocytes, dependent on the affinity of TCRαβ for self peptide-loaded major histocompatibility complex (self pMHC) in thymic stromal cells. During the process, premature thymocytes with TCR that fail to recognize the self pMHC die for the lack of TCR signaling (death by neglect), whereas thymocytes with TCR that binds self pMHC with high affinity to be eliminated by negative selection. The only thymocytes that moderately recognize self-pMHC with the appropriate strength of TCR signal can undergo positive selection (Fu et al., 2012; Klein et al., 2014). When the strength and duration of signaling induced by TCR/ligand interaction in the thymus not only determines which cells will be positively selected, but also guides them to differentiate into either CD4 T cells or CD8 T cells.

Differentiation of T cells is continued in the periphery, which occurs upon stimulation with antigen, antigen-presenting cells, and cytokines (Bosselut, 2004; Singer, 2002). The most proximal event associated with TCR ligation is the activation of tyrosine kinases, including Zap70, Itk, Lck and Fyn. After ligation of the TCR, the Src-family kinase Lck phosphorylates tyrosine residues in immunoreceptor tyrosine-based activation motifs of the invariant signaling protein CD3ζ

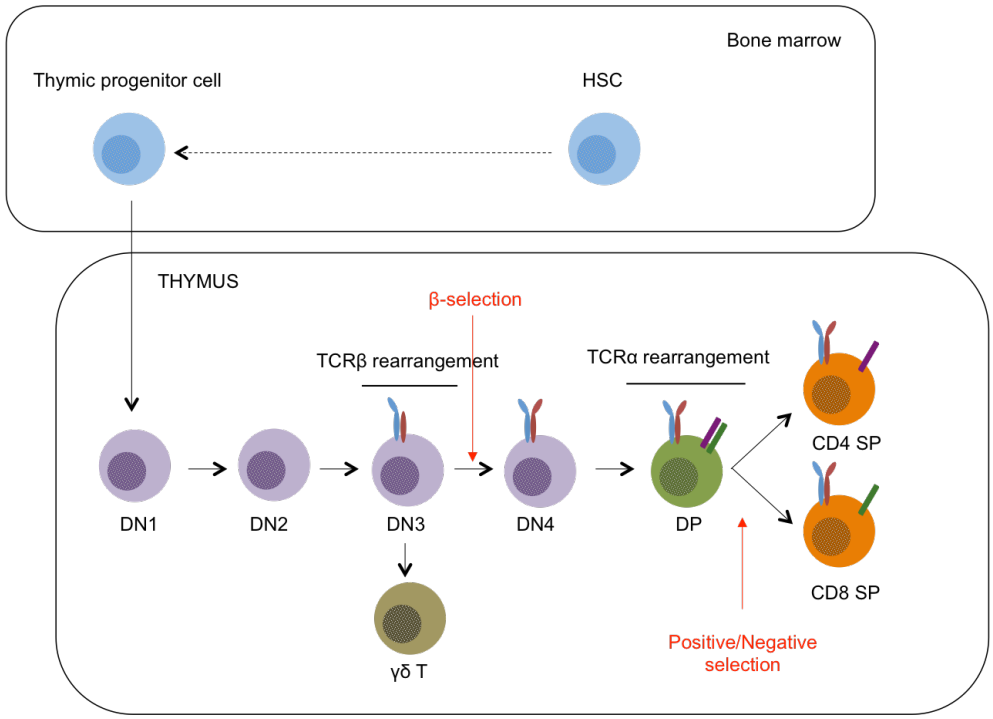

Figure 1. Overview of thymocyte development

complex, which is followed by the recruitment and activation of Zap70. Activated Zap70 phosphorylates the adaptor Lat, which results in the formation of the multimolecular signalosome complex known as the 'Lat signalosome', which includes PLC-γ1, Grb2, Vav, Gads, SLP-76 and Themis (Brockmeyer et al., 2011; Werlen and Palmer, 2002). Recruitment to the Lat signalosome activates these signaling molecules, leading to subsequent activation of signaling cascades, including the mitogen-activated protein kinase Erk and calcium mobilization, which are hallmarks of TCR signaling and are critical for the survival, differentiation and maturation of thymocytes (Kane and Hedrick, 1996). Deletion or manipulation of intracellular kinases such as Lck, Zap70, Tec-family kinases and Erk results in defective thymocyte selection.

I-2. SWI/SNF chromatin remodeling complexes

The dynamic modification of chromatin structure allows access of condensed DNA to the transcription machinery proteins for gene expression. The chromatin status that is accessible or inaccessible to the transcription machinery is very closely related to the gene expression (Razin, 1998). Chromatin remodeling complex is a fundamental regulator involved in all major reactions with chromatin substrate. Chromatin remodeling complexes use the energy of the ATP hydrolysis to change the condensed chromatin structure (Becker and Hörz, 2002). The family of

ATP-dependent chromatin remodeling complexes can be subdivided into five different classes: SWI/SNF, ISWI, NuRD/Mi2/CHD, INO80, and SWR1. Because each complex bears unique flanking domains, although all complexes share a conserved ATPase domain, the chromatin complexes are distinguishable: That is, BRG or BRM, SNF2H, and Mi2, for BAF, ISWI and NURD complexes, respectively (Flaus et al., 2006; Lusser et al., 2005).

The SWI/SNF complex is highly conserved multi-subunit complex (Kingston and Narlikar, 1999; Muchardt and Yaniv, 1999), which was first discovered in yeast (Winston and Carlson, 1992). Human SWI/SNF complexes contain a single ATPase, either BRM (encoded by the *SMARCA2* gene) or BRG1 (*SMARCA4*), and three main core subunits: BAF155 (*SMARCC1*), BAF170 (*SMARCC2*), and BAF47 (*SMARCB1*). These complexes divided into two main types, depending upon their subunit composition: BAF complexes contain either BAF250a (*ARID1A*) or BAF250b (*ARID1B*) subunits; PBAF complexes contain BAF180 (*PBRM1*) and BAF200 (*ARID2*) subunits. In addition to these core subunits, SWI/SNF complexes contain 7 to 15 accessory subunits. Subunit diversity of the mammalian SWI/SNF complex suggests different complexes might have tissue-specific roles (Wang et al., 1996).

The accessibility of the SWI/SNF complex to chromatin is regulated by TCR signaling. TCR signaling induces the rapid association of the SWI/SNF complex with chromatin in peripheral T cells (Zhao et al.,

1998). Polycomb repressive complex 1 (PRC1) has been shown to negatively regulate chromatin accessibility of the SWI/SNF complex (Shao et al., 1997). The chromatin remodeling activity of the SWI/SNF complex is also regulated by phosphorylation in cellular processes, particularly during mitosis. BRG1 and hBRM undergo phosphorylation on entry into mitosis, which might be used as a switch to modulate the SWI/SNF complex activity during cell cycle (Muchardt et al., 1996; Reyes et al., 1997). The stability of the SWI/SNF complex is reported to be regulated by ubiquitylation. Unkempt, a RING finger protein, binds to BAF60b and induces its ubiquitylation and proteosomal degradation (Lorès et al., 2010). BAF57 is also ubiquitylated and degraded by the E3 ubiquitin ligase, Thyroid hormone receptor interacting protein 12 (TRIP12). BAF155 blocks the interaction between BAF57 and TRIP12 and, thus, protects BAF57 from degradation (Keppler and Archer, 2010). These results suggest that protein degradation by the ubiquitylation pathway regulates the quality and functional fidelity of the SWI/SNF complex.

I-3. SRG3, a scaffold subunit of the SWI/SNF complex

Srg3 (*Swi3-related gene*) is a murine homolog of yeast *Swi3*, Drosophila *Moira*, and human *Baf155* (Sudarsanam and Winston, 2000), was initially isolated as a highly expressed gene in thymus but not in

spleen (Jeon et al., 1997). It was reported that BAF155 was essential for the chromatin remodeling function of BRG1 (Kadam, 2000). The expression level of SRG3 is critical for glucocorticoid (GC) sensitivity in thymocytes (Han et al., 2001; Jeon et al., 1997; Ko et al., 2004a). In addition, during positive selection of thymocytes, TCR signaling induces the expression of Id3 protein, which blocks the activity of E2A protein and thereby downregulates the SRG3 expression (Ko et al., 2004a; Ko et al., 2004b). SRG3 also regulated by Notch signaling during DP thymocyte maturation (Choi et al., 2001; Jang et al., 2006) and Nitric oxide (NO) signaling represses SRG3 expression via the inactivation of the transcription factor SP1's DNA binding activity to the SRG promoter (Jeong et al., 2004).

Previous studies have shown that SRG3 has a scaffold function in stabilizing the SWI/SNF by interacting directly with the core subunits BRG1, SNF5, and BAF60a (Sohn et al., 2007). SRG3 interacts with BRG1 through its highly conserved SANT (SWI3, ADA2, N-CoR, and TFIIIB) domain (Aasland et al., 1996). It also interacts with SNF5 and BAF60a through its conserved SWIRM (SWI3, RSC8, and MOIRA) domain. SRG3 also interacts with other components of the SWI/SNF complex and stabilizes them by attenuating or blocking their proteasomal degradation (Sohn et al., 2007). It has also been reported that MOIRA and BAF155 interact with BRM and BAF57, respectively (Crosby et al., 1999; Keppler and Archer, 2010). These results suggest that SRG3 is a

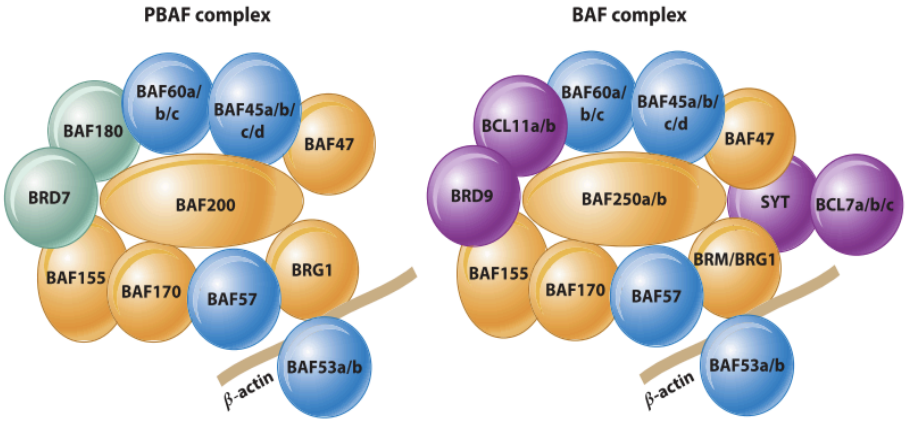

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Figure 2. The SWI/SNF chromatin remodeling complex

scaffold that interacts physically with other components of the SWI/SNF chromatin remodeling complex and stabilizes them.

I-4. The function of SWI/SNF complex in thymocyte development

In the past few years, many studies have shown to address the roles of the SWI/SNF complexes in the immune system from lymphocyte development to immune responses. T-cell-specific inactivation of SWI/SNF complexes indicates that they are essential for the developmental transitions of thymocytes. The SWI/SNF complexes serve a central role in the regulation of genes involves in hematopoietic lineage specification. For example, Interaction of SWI/SNF complexes with Ikaros, a zinc finger transcription factor that is important in lymphocyte development, provide a relation between chromatin modification and hematopoietic cell fate decisions (Georgopoulos, 2002). The sustained expression of dominant negative mutant of *BAF57* resulted in the de-repression of CD4 and the inactivation of CD8 expression in immature T cell subsets (Chi et al., 2002). The downregulation of IL-2Rα (CD25) transcription at DN stage was correlated with dissociation of histone acetylase p300 and BRG1 from its promoter (Yeh et al., 2002). In addition, the deficiency of Brg1 led to the impairment of CD4/CD8 expression in DN cells and reduction of cell viability of DP cells (Chi et al., 2003; Gebuhr et al., 2003). Brg1 is also critical for innate and adaptive

immune responses. BRG1 selectively activates a subset of interferon-α-inducible genes and functions downstream of TCR signaling to decondense chromatin during T-cell activation (Huang et al., 2002; Zhao et al., 1998). These results implicated that the SWI/SNF complex mediates the expression of genes involved in thymocyte development and immune responses.

I-5. The Ubiquitin-Proteasome system

Ubiquitin is a highly conserved 76 amino acids polypeptide in eukaryotes. The cascade of E1, E2 and E3 enzymes conjugates ubiquitin to lysine residues (Pickart, 2001). In humans, there are two E1 enzymes, about forty E2 enzymes and hundreds of E3 enzymes. A protein can be modified on one lysine residue with a single ubiquitin (mono-ubiquitylation) or with a chain of ubiquitin (poly-ubiquitylation). Lysine 48 (K48)-linked poly-ubiquitylation usually targets proteins for proteasomal degradation, whereas K63-linked poly-ubiquitylation is implicated in many signal transduction cascades, such as DNA repair and protein kinase activation (Chen et al., 2009). The proteasome is a complicated molecular machine, which perform to degrade proteins following their conjugation to ubiquitin (Herrmann et al., 2007; Welchman et al., 2005). The proteasome has a proteolytic subunit and an ATP-dependent regulatory subunit, functionally linked by a gated protein

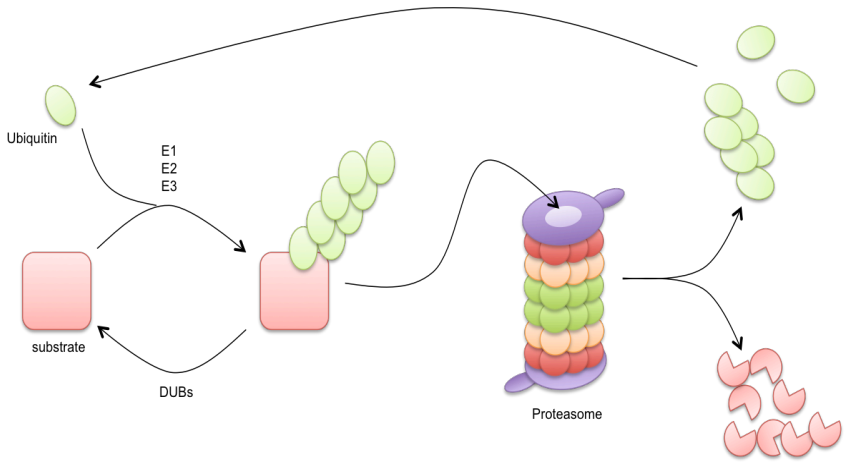


Figure 3. The Ubiquitin-Proteasome system

translocation channel. The proteasome exists in multiple forms, but contains two major assemblies, the 28-subunit core particle (CP, also known as the 20S particle) and a regulatory particle (RP, also known as the 19S particle and PA700). The CP is a barrel-like structure whose subunits are arranged in four stacked seven-membered rings (Borissenko and Groll, 2007). The proteolytic active sites of proteasome are imbedded within the internal space of the CP. The poly-ubiquitylated proteins are recognized by the RP, and then enter the inside of the CP in contact with proteolytic active sites and degrade to peptide fragments. In eukaryotes, the ubiquitin-proteasome degradation pathway is the major proteolytic system with functions in diverse biological processes such as cell cycle, apoptosis, inflammation, transcription, signal transduction and protein quality control (Huang and D'Andrea, 2006; Li et al., 2009; Mukhopadhyay and Riezman, 2007).

I-6. CHFR, an E3 ubiquitin ligase

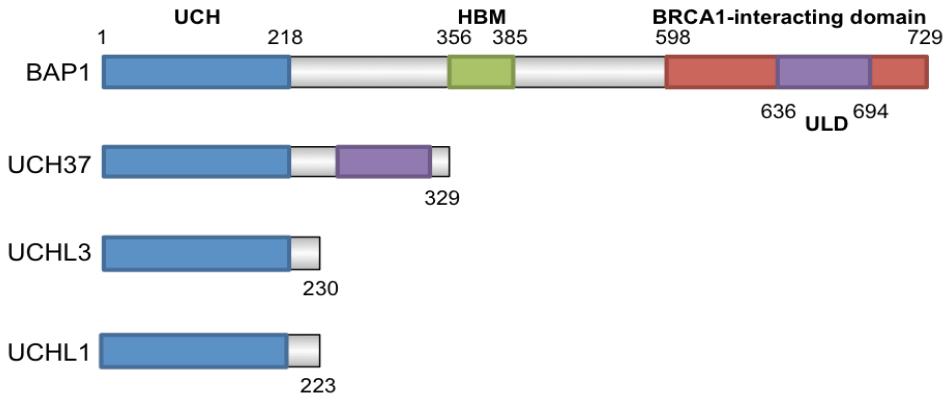
Specificity in the choice of ubiquitylation substrate and the type of ubiquitin chain is largely determined by E2 and E3 enzymes (Ye and Rape, 2009). The E3 ligases, which specifically binds a protein substrate and facilitates the transfer of ubiquitin from E2 to a lysine residue in the substrate (Pickart, 2001; Weissman, 2001), are classified into two families: HECT (homologous to the E6-associated protein C terminus),

RING (Really Interesting New Gene)-finger. CHFR (Checkpoint with FHA and RING finger domains) is one of the E3 ubiquitin ligases, which is known to be a tumor suppressor and play an essential role in cell cycle control and tumorigenesis. CHFR is inactivated in various tumors, including colorectal, gastric, lung, and breast (Ogi et al., 2014; Satoh et al., 2003; Toyota and Suzuki, 2010). CHFR controls Aurora A protein levels through ubiquitylation, consequently, overexpression of Aurora A in cancer may be explained in part by inactivation of CHFR. CHFR knockout mice develop solid tumors and lymphomas, and embryonic fibroblasts from CHFR knockout mice show a mitotic checkpoint defect that causes chromosomal instability (Yu et al., 2005). In addition, CHFR interacts with helicase-like transcription factor (HLTF), which belongs to the SWI/SNF family and is silenced by promoter hypermethylation in colorectal, gastric, and cervical cancers (Hamai et al., 2003), and negatively-regulates its stability and functions by ubiquitylation (Kim et al., 2010).

I-7. BAP1, a deubiquitinating enzyme

Ubiquitylation can be reversed by deubiquitinating enzymes (DUBs), which form a large group of proteases. DUBs can reverse ubiquitylation by cleaving the peptide or isopeptide bond between ubiquitin and its substrate protein. In humans, there are almost one

(A)



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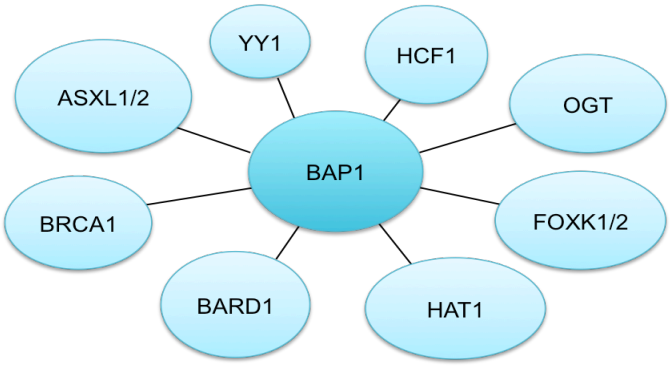


Figure 4. The structure of deubiquitinase BAP1 and its protein partners

UCH, Ubiqiutin carboxyl-terminal hydrolase domain; HBM, HCF-1- binding motif; ULD, UCH37-like domain.

hundred putative DUB genes, which can be classified into two main classes: cysteine proteases and metalloproteases (Finley, 2009). There are four main superfamilies of cysteine protease DUBs, the ubiquitin-specific protease (USP/UBP) superfamily: the ovarian tumor (OTU) superfamily, the Machado-Josephin domain (MJD) superfamily and the ubiquitin C-terminal hydrolase (UCH) superfamily. The JAB1/MPN/Mov34 metalloenzyme (JAMM) domain superfamily proteins are zinc-dependent metalloprotease family (Amerik and Hochstrasser, 2004). The deubiquityation is a highly regulated process that has been implicated in numerous cellular functions, including cell cycle regulation, proteasome- and lysosome-dependent protein degradation, gene expression and DNA repair (Daniel and Grant, 2007; Song and Rape, 2008). A number of pathogenic microorganisms have acquired genes encoding DUBs suggesting that disruption of ubiquitylation in the host cell may confer a selective advantage for these bacteria and viruses (Lindner, 2007; Rytkönen and Holden, 2007). Furthermore, mutations in several deubiquitinating enzymes have been linked to disease ranging from cancer to neurological disorders (Fischer, 2003).

BRCA1-associated protein-1 (BAP1) is a nuclear deubiquitinating enzyme (DUB) that was identified as breast cancer type 1 susceptibility protein (BRCA1)-interacting ubiquitin carboxyl-terminal hydrolase (UCH) that is involved in the removal of ubiquitin from proteins (Jensen et al., 1998; Nishikawa et al., 2009). Many studies have

shown that BAP1 roles as a tumor suppressor (Jensen and Iii, 1999). BAP1 was inactivated in lung carcinoma, breast cancer cell lines, and renal cell cancers (Abdel-Rahman et al., 2011; Coupier et al., 2005; Jensen et al., 1998; Ventii et al., 2008). Somatic and germline BAP1 mutations also increased the possibility of mesothelioma and uveal melanoma (Abdel-Rahman et al., 2011; Goldstein, 2011; Testa et al., 2011). In addition, RNA interference (RNAi)-mediated depletion of BAP1 induced cell-cycle progression defects such as the S-phase retardation (Nishikawa et al., 2009).

The *Drosophila* gene *calypso*, a homologue of the human *BAP1* gene, is a Polycomb group (PcG) gene, which is transcriptional repressor that regulates cell differentiation and development (Scheuermann et al., 2010). Calypso interacts and makes a complex with Asx (Additional sex combs) that is homologous of the human Asxl1 (Additional sex combs-like 1), where the complex was named Polycomb repressive deubiquitinase (PR-DUB). The complex binds at PcG target genes in *Drosophila*, and then, removes mono-ubiquitin from histone H2A, which leads the chromatin remodeling and gene silencing (Figure 5A). In humans, BAP1 interacts with Additional sex combs-like 1 (ASXL1) through its carboxyl-terminal and deubiquitinates histone H2A and regulates expression of target genes. It had been observed that the ubiquitin of H2A level was subsequently changed when the level of BAP1 was altered in renal cancer cells (Peña-Llopis et al., 2012). BAP1 regulate

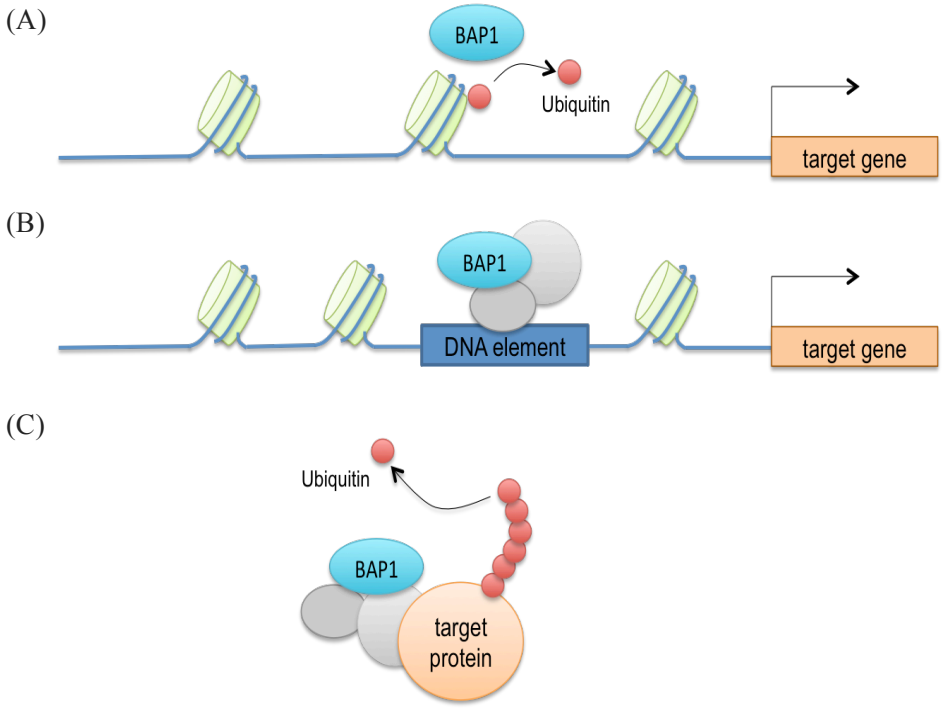


Figure 5. The mechanism of BAP1 functions

gene transcription via association with other protein partners (Figure 5B). BAP1 interacts with the zinc fingers of YY1 through its coiled- coil motif and it is recruited together with HCF1 to the promoter of the gene that encodes a component of the mitochondrial respiratory chain (Yu et al., 2010). Also, deubiquitylating activity of BAP1 controls stability of target proteins (Figure 5C). The transcriptional regulator host cell factor-1 (HCF-1) and its interacting partner O-linked N-acetylglucosamine transferase (OGT), which itself positively regulates HCF-1 activity by glycosylation (Dey et al., 2012), are stabilized through deubiquitylation by BAP1. This complex plays a critical role in glucose sensing. Levels of the promoter of gluconeogenesis, peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α, are increased in euglycemic conditions. It is proposed that N-acetylglucosylation of PGC-1α by HCF-1/OGT promotes its stability, through the recruitment of BAP1 deubiquitylating activity (Yang et al., 2012).

I-8. The function of BAP1 during hematopoiesis

Although the molecular mechanism still remains elusive, several evidences have been shown that BAP1 is also involved in hematopoiesis. The deletion of BAP1 in murine model resulted in embryonic lethality (Dey et al., 2012). The inducible deletion of BAP1 using creERT2 system showed severe splenomegaly resulted from extramedullary hematopoiesis

and expansion of the myeloid lineage. Total leukocytes cellularity was increased because of monocytosis and neutrophilia. Overall, BAP1 deletion showed the features of myeloprolitive and myelodysplastic disorder that resembles human chronic myelomonocytic leukemia (CMML) (Dey et al., 2012). Furthermore, the systemic deletion of BAP1 at the hematopoietic stem cells (HSCs) led to an increase in the number of LSK (lineage-negative, Sca-1^{+}, c-Kit^{+}) and myeloid progenitor cells in the bone marrow. BAP1-deficient bone marrow progenitors exhibited decreased self- renewal as assayed by *in vitro* colony-forming assays and competitive transplantation assays using whole bone marrow mononuclear cells depleted of BAP1 before transplantation. Nevertheless, much remains unknown about the roles of BAP1 during thymocytes development in the thymus.

CHAPTER II.

MATERIALS AND METHODS

Plasmid constructs

For cloning of plasmids used in transfection, the cDNAs for murine SRG3, BRG1, SNF5, and BAF60a were inserted into the pCAGGS or pCAGGSBS vector with N-terminal FLAG or Myc tag. The mutants of SNF5 were PCR-amplified with appropriate primers for FLAG-SNF5 (1–319), -SNF5 (1–245), and -SNF5 (1–185 + Rpt2) and inserted into the pCAGGSBS vector. Each construct was tagged with N-terminal FLAG epitope. The pcDNA3-Myc-CHFR plasmid construct and HA-Ubiquitin plasmid construct were generously provided by JH, Seol (Seoul National University, Seoul, Korea) and CH, Chung (Seoul National University, Seoul, Korea), respectively.

Cell culture and transient transfection

COS-1 cells and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, WelGENE) containing 10% fetal bovine serum (FBS, WelGENE). NIH3T3-U6 and NIH3T3-U6-shSRG3 stable cell lines were maintained in DMEM containing 10% bovine calf serum (BCS, HyClone). All the transfection experiments were performed with Lipofectamine 2000 (Invitrogen), CaPO4-method or polyethyleneimine (PEI) according to the manufacturer's instructions. All cells were split into 60 mm dishes to 70–80% confluency at 24 h before transfection. Appropriate control empty vectors were supplemented to adjust the total amounts of DNA in each experiment. Cells were harvested after 48 h of

incubation. Before the harvesting, the cells were treated with 20 μM of MG132 (A.G. Scientific, INC.) for 6 h.

Immunoprecipitation, and immunoblotting

For immunoprecipitation experiments, cells were lysed in buffer X (100 mMTris–Cl pH 8.5, 250 mM NaCl, 1% NP-40, 1 mM EDTA, 2 mg/ml BSA) and the proteins were immunoprecipitated with specific antibodies as previously described. For Immunoblot analysis, cells were lysed in RIPA buffer in the presence of protease inhibitors. Proteins were subjected to SDS–PAGE and transferred to immobilon-P membrane (Millipore). Blotted proteins were detected using antibodies of anti-FLAG (M2, Sigma), anti-Myc (9E10, Roche Applied Science), anti-HA (HA-7, Sigma), anti-BAP1 (H-300, Santa Cruz) and anti-β-actin (AC-15, Sigma) were purchased commercially. Antisera against SRG3 were raised from rabbits in our laboratory.

In vitro ubiquitylation assay

For in vitro ubiquitylation assay, FLAG-BRG1, FLAG-SNF5 or FLAG-BAF60a translated in vitro by using TNT T7 quick coupled system (Promega, L1170) was incubated at 37 °C for 0 or 30 min with E1 (0.5 μg), E2 (UBCH5a, 0.5 μg), ubiquitin (10 μg), 1 mM DTT, and 5 mM ATP in the presence or absence of E3 (His-CHFR, 7 μg) as previously described. After the indicated times, each sample was analyzed by

immunoblotting with anti-SNF5, -BAF60a, or -BRG1 antibodies.

Mice

Mice with loxP sites between 12 and 13 exon of *Bap1* were provided by Soo-Jong Um (Sejong University, Seoul, Korea), which was crossed with Flp-transgenic mice to remove the neo selection cassette. These $Bap1^{fl/fl}$ mice were crossed to the Lck-Cre or CD4-Cre mice. Lck-Cre, CD4-Cre and HY TCR transgenic mice were purchased from Taconic. RAG knockout mice were purchased from the Jackson Laboratory. All mice were bred and maintained in pathogen-free barrier facilities at Seoul National University and used according to protocols approved by Institutional Animal Care and Use Committees (IACCU) of Seoul National University.

Flow cytometry and cell sorting

Single-cell suspensions from the thymus, spleen and lymph node were prepared by passing through the cell strainer to get rid of cell debris and surface stained as described (ref). To deplete the red blood cells, an equal volume of ACK RBC lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) was added to the cells, and then incubated on room temperature for 5 min. Then up to 10 ml of 1X PBS was added to stop the lysis reaction. Cells were stained with antibodies in various combinations in 1X PBS. Flow cytometry analyses were performed using

FACSCanto II (BD Bioscience). The antibodies used are as follows: Anti-CD4-PE (GK1.5, BD), -CD4-PE-Cy7 (GK1.5, eBioscience), -CD8-APC-Cy7 (53-6.7, eBioscience), -CD25-PE (PC61, BD), -CD25-PE-Cy7 (PC61.5, eBioscience), -CD44-FITC (IM7, eBioscience), -CD69-Biotin (BD), -CD44-Biotin (IM7, eBioscience), -TCRβ-FITC (H57-597, BD), -HY-APC (T3.70, eBioscience), -CD24-PE-Cy7 (M1/69, BioLegend), -CD62L-FITC (MEL-14, BD), -mouse IgG1-PE (A85-1, BD), -IgG-FITC (BD), -Streptavidin-PerCP (BD). FACS AriaII (BD Bioscience) was used for sorting of cells.

Hematoxylin and eosin staining

Thymi were fixed in 4% paraformaldehyde and 0.05% glutaraldehyde in phosphate-buffered saline (PBS) for 2h at 4°C, embedded in paraffin, and serially sectioned (5μm). Some sections were stained with routine hematoxylin-eosin.

Quantitative RT-PCR analysis

Total RNA was purified from cells with TRIzol® reagent according to the manufacturer's instructions (Invitrogen). RNA was reverse transcribed with SuperScript III (Invitrogen) and diluted cDNAs were analyzed by quantitative real-time PCR. SYBR Green PCR mix (Applied Biosystem) was used for quantitative RT PCR and the results

were quantified with StepOnePlus (Applied Biosystem). The showed data were relative values. Primer sequences are available upon request.

CD69-upregulation assay

Sorted naive splenocytes were stimulated for 5 hours with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) at 37°C. Cells were stained with anti-CD69 antibody and anti-CD4 or anti-CD8 antibody and analyzed after gating $CD4^+$ or $CD8^+$ cells.

Ca^{2+} flux

A total of 5 x 10^6 Thymocytes were labeled in RPMI medium containing Fluo-3 (Invitrogen), in the presence of Pluronic (Invitrogen) for 30 min at 37°C. Cells the washed with RPMI twice. After adding anti-CD3ε-biotin antibody, cells were incubated on ice for 15min, washed twice with RPMI medium, and resuspended in 1x HBS solution. Cells were prewarmed to 37°C before analysis and then collected on a FACSCanto II (BD bioscience) for 30 sec to establish a baseline for unstimulated cells and the change in the Ca^{2+} flux was monitored for the following 5 min. For cell stimulation, prewarmed streptavidin (10 μg/ml) was added to cells. Mean fluorescence ration was calculated with FlowJo (Tree star).

Intracellular staining

After surface staining on CD25, CD44, CD4 and CD8, single cell suspension of total thymocytes was fixed and permeabilized with Fixation/Permeabilization buffer (BD Bioscience) according to the manufacturer's instructions. Cells were stained with anti-TCRβ antibody (BD Bioscience). For phosphor-ERK staining, cells were stained with phospho-ERK specific antibody, followed by staining with PE-conjugated anti-mouse IgG1 antibody. Cell events were collected by using a FACSCanto II (BD bioscience), and the data was analyzed with FlowJo (Tree star).

Statistical analysis

Prism software (Graphpad) was used for all statistical analysis. Two-tailed *Student's t-tests* were used to calculate p values where indicated.

CHAPTER III.

RESULTS

III-1. SRG3 stabilizes the SWI/SNF complex by blocking CHFR mediated ubiquitylation and degradation

Major components of the SWI/SNF complex interact with CHFR

BRG1, SNF5, and BAF60a are degraded by a proteasome-mediated degradation pathway (Sohn et al., 2007). The degradation of BRG1, SNF5, and BAF60a was inhibited by treatment with the potent proteasome inhibitor MG132, and SNF5 protein was poly-ubiquitylated (Sohn et al., 2007). It was tested whether BAF60a and BRG1 are also poly-ubiquitylated in the presence of MG132 (Figure 6A and B). Significant increases in BAF60a-ubiquitin and BRG1-ubiquitin conjugates were detected in the presence of MG132. These results show that BAF60a and BRG1, as well as SNF5, are ubiquitylated and regulated by a proteasome-mediated degradation pathway.

CHFR is known to function as a mitotic checkpoint protein and as an ubiquitin ligase for HLTF (Ding et al., 1996; Kim et al., 2010). These results suggested the possibility that CHFR may function as an E3 ubiquitin ligase for the components of the SWI/SNF complex. Therefore, It was analyzed the interaction between the components of the mammalian SWI/SNF complex and CHFR by immunoprecipitation. The expression vectors of FLAG-tagged BRG1, SNF5, BAF60a, or SRG3 were co-transfected with Myc-CHFR expression vector into COS-1 cells,

and cell lysates were immunoprecipitated with anti-FLAG or anti-Myc antibodies. As shown in Figure 7A–D, BRG1, SNF5, and BAF60a were co-immunoprecipitated with CHFR, whereas SRG3 did not interact with CHFR at all. These results suggest that BRG1, SNF5, and BAF60a, but not SRG3, are the substrates of CHFR for ubiquitylation.

CHFR induces ubiquitylation of major components of the SWI/SNF complex

I further examined whether CHFR actually induces ubiquitylation of the components of the SWI/SNF complex, both *in vitro* and *in vivo*. For the *in vitro* ubiquitylation assay, FLAG-tagged BRG1, SNF5, or BAF60a, which were translated *in vitro*, incubated with or without CHFR for the indicated times. As shown in Figure 8A–C, the ubiquitylation of BRG1, SNF5, or BAF60a increased when these proteins were incubated with CHFR for 30 minutes. To confirm that CHFR induces ubiquitylation *in vivo*, 293T cells were co-transfected with FLAG-SNF5 and HA-Ubiquitin expression vectors, with or without Myc-CHFR expression vector, and cell lysates were immunoprecipitated with anti-FLAG antibody (Figure 9). In the presence of CHFR, the SNF5-ubiquitin conjugates significantly increased, indicating that CHFR induces the ubiquitylation of SNF5 *in vivo*.

Next, It was investigated whether CHFR can increase the degrad-

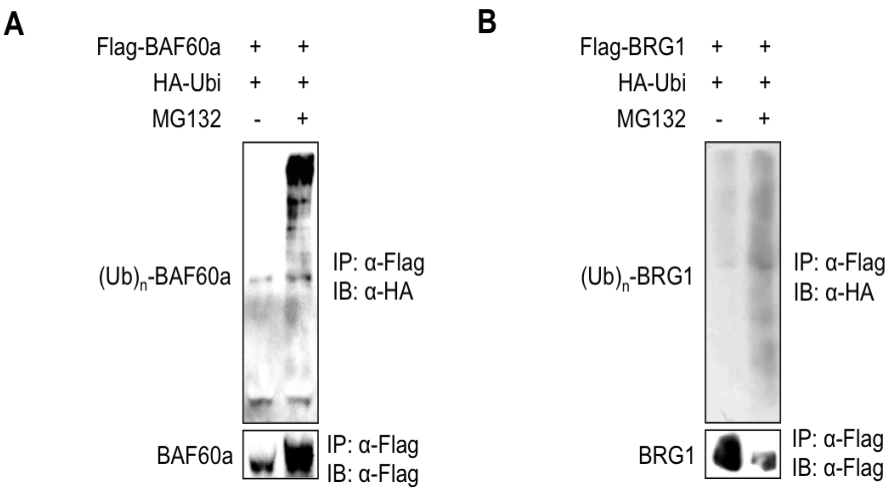
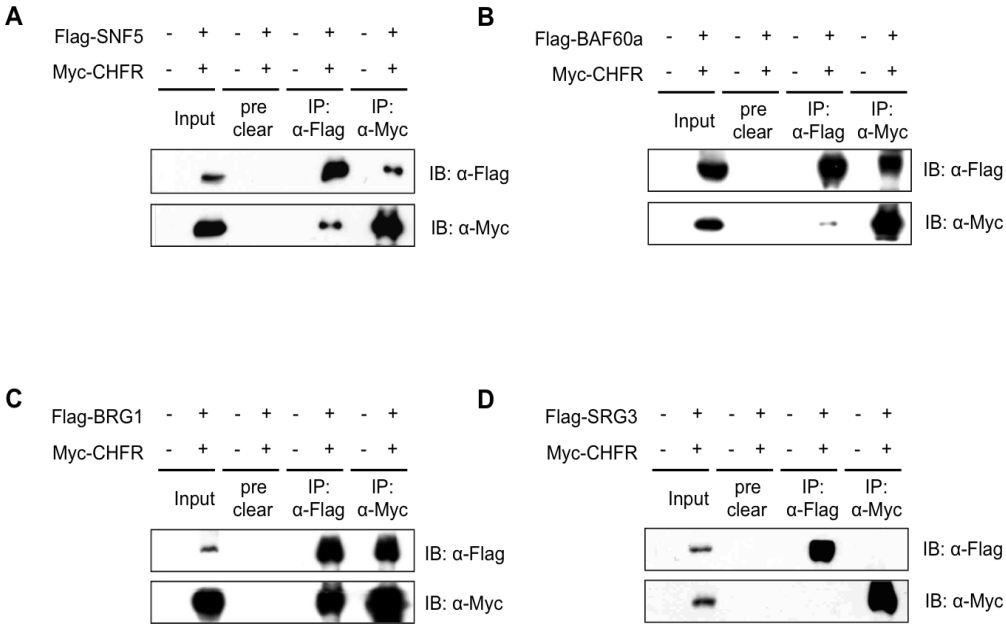


Figure 6. The components of the SWI/SNF complex are poly-ubiquitinated by the proteasome-mediated pathway.

COS-1 cells were co-transfected with FLAG-BAF60a (A) or FLAG-BRG1 (B) expression vectors along with HA-Ubiquitin expression vector. After 42 hours of incubation, cells were treated with MG132 (20 μM) for 6 hours. Whole cell lysates were immunoprecipitated with anti-FLAG antibody and subjected to immunoblot analysis with anti-HA antibody (upper panel). The membranes were stripped after analysis and immunoblotted again with anti-FLAG antibody (lower panel).

Figure 7. The major components of the SWI/SNF complex interact with CHFR.

FLAG-SNF5 (A), FLAG-BAF60a (B), FLAG-BRG1 (C) or FLAG-SRG3 (D) expression vectors were co-transfected into COS-1 cells with Myc-CHFR expression vectors. Whole cell lysates were immunoprecipitated with anti-FLAG or anti-Myc antibodies and subjected to immunoblot analysis with anti-Myc or anti-FLAG antibodies, respectively. The cells transfected with an empty vector were used as control (labeled as 'Mock').


A
Flag-SNF5 - + - + - + - +
Myc-CHFR - + - + - + - +
Input
pre clear
IP: α-Flag
IP: α-Myc
IB: α-Flag
IB: α-Myc
B
Flag-BAF60a - + - + - + - +
Myc-CHFR - + - + - + - +
Input
pre clear
IP: α-Flag
IP: α-Myc
IB: α-Flag
IB: α-Myc
C
Flag-BRG1 - + - + - + - +
Myc-CHFR - + - + - + - +
Input
pre clear
IP: α-Flag
IP: α-Myc
IB: α-Flag
IB: α-Myc
D
Flag-SRG3 - + - + - + - +
Myc-CHFR - + - + - + - +
Input
pre clear
IP: α-Flag
IP: α-Myc
IB: α-Flag
IB: α-Myc

ation of its substrates, BRG1, SNF5, and BAF60a. 293T cells were co-transfected with FLAG-BRG1, -SNF5, or -BAF60a expression vector together with the Myc-CHFR expression vector (Figure 10A–D). CHFR downregulated the stability of these components in a dose-dependent manner. In the presence of MG132, the protein levels were restored to those at which CHFR was not overexpressed. These findings indicate that CHFR increases the degradation of BRG1, SNF5, and BAF60a through the ubiquitylation-proteasome-mediated degradation pathway. However, SRG3 protein was not affected by CHFR overexpression at all. As described previously, unlike other components, SRG3 did not interact with CHFR. Thus, CHFR regulates the stability of the components of the SWI/SNF complex by inducing ubiquitylation but does not affect SRG3 level. In addition, the SNF5-ubiquitin conjugates significantly increased in the presence of MG132 with CHFR *in vivo*. These results suggest that CHFR enhances the degradation of the components of the SWI/SNF complex by inducing their poly-ubiquitylation.

SRG3 can stabilize the SWI/SNF complex by interfering with CHFR

Since SRG3 and CHFR have opposite roles in stabilizing the subunits of the SWI/SNF complex, it is likely that SRG3 inhibits the activity of CHFR for regulation of protein stability of those components.

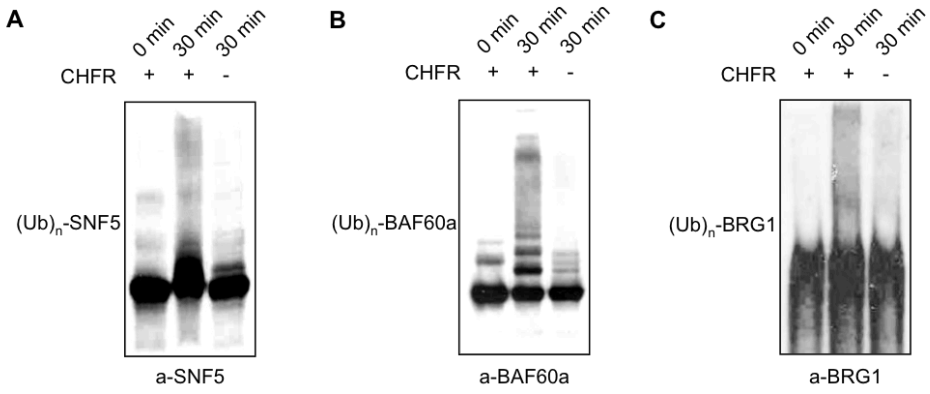


Figure 8. CHFR enhances the degradation of the SWI/SNF complex *in vitro* by inducing its ubiquitination.

For *in vitro* ubiquitination assay, *in vitro* translated FLAG-SNF5 (A), FLAG-BAF60a (B) or FLAG-BRG1 (C) were incubated with E1, E2 (UBC-5Q), ubiquitin, ATP, DTT, protease inhibitor, and His-CHFR for the indicated times at 37°C. After incubation, the samples were immunoblotted with anti-SNF5, -BAF60a, or -BRG1 antibodies. To verify this, BRG1, SNF5, or BAF60a expression vectors were co-transfected together with increasing amounts of CHFR expression vector

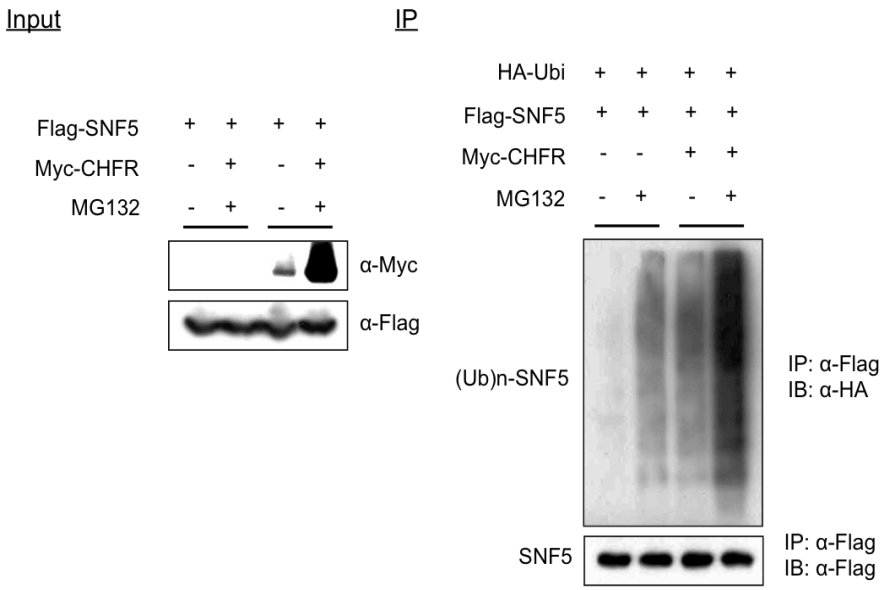
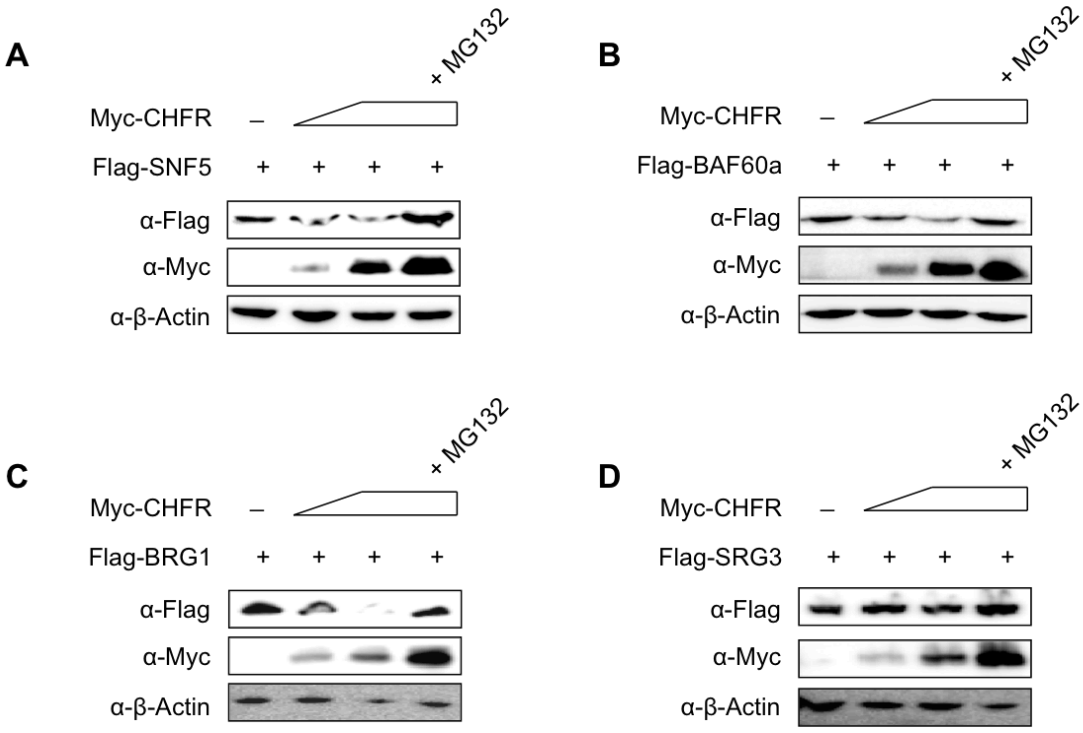


Figure 9. CHFR induces the ubiquitination of SNF5 *in vivo*.

293T cells were co-transfected with FLAG-SNF5 and HA-Ubiquitin expression vectors, and treated with MG132 (20 μM) or vehicle for 6 hours. After 48 hours of incubation, whole cell lysates were subjected to SDS-PAGE and immunoblotted with anti-Myc or anti-FLAG antibodies (left panel). Also, the cell lysates were immunoprecipitated with anti-FLAG antibody and subjected to SDS-PAGE, and immunoblotted with anti-HA antibody (right, upper panel) or anti-FLAG antibody (right, lower panel).

Figure 10. CHFR increases the degradation of the components of the SWI/SNF complex through the ubiquitination-proteasome-mediated pathway.

293T cells were co-transfected with FLAG-SNF5 (A), FLAG-BAF60a (B), FLAG-BRG1 (C), or FLAG-SRG3 (D) expression vectors along with increasing amount of Myc-CHFR expression vector. Cells were treated with MG132 (20 μM) for 6 hours and whole cell lysates were analyzed by immunoblot analysis with anti-Myc and anti-FLAG antibodies.


A
+ MG132
Myc-CHFR –
Flag-SNF5 + + + +
α-Flag
α-Myc
α-β-Actin
B
+ MG132
Myc-CHFR –
Flag-BAF60a + + + +
α-Flag
α-Myc
α-β-Actin
C
+ MG132
Myc-CHFR –
Flag-BRG1 + + + +
α-Flag
α-Myc
α-β-Actin
D
+ MG132
Myc-CHFR –
Flag-SRG3 + + + +
α-Flag
α-Myc
α-β-Actin

and a fixed amount of SRG3 expression vector into 293T cells and analyzed the protein levels of each component by immunoblot analysis. As shown in Figure 11A–C, the protein levels of BRG1, SNF5, and BAF60a were downregulated by CHFR expression but were maintained in the presence of SRG3, although the level of CHFR was not significantly changed. These results suggest that the degradation of the components of the SWI/SNF complex induced by CHFR is inhibited by SRG3 expression. It was further examined whether the protein stability and ubiquitylation of BAF60a is affected by *Srg3* knockdown. The established NIH3T3 cell line stably expressing small hairpin RNA against *Srg3 (shSRG3)* and its control cell line (Ahn et al., 2011) were co-transfected with FLAG-BAF60a expression vector and HA-Ubiquitin expression vector (Figure 12). BAF60a level was decreased by knockdown of *Srg3* and restored by MG132 treatment. Cell lysates were immunoprecipitated with anti-FLAG antibody and then immunoblotted with anti-HA antibody for detecting ubiquitylated BAF60a proteins. In the presence of MG132, the ubiquitylation of BAF60a was increased by *Srg3* knockdown. Thus, SRG3 stabilizes BAF60a by inhibiting the ubiquitylation of BAF60a, which is induced by CHFR.

Rpt2 region of SNF5 is the CHFR-interacting region

Alignment analysis of SNF5 and its homologs revealed that SNF5 has 2 highly conserved domains, namely, repeat 1 (Rpt1) and repeat 2 (Rpt2), and 1 moderately conserved domain, namely, homology region 3 (HR3) (Craig et al., 2002; Morozov et al., 1998). To identify which regions of SNF5 are important for the interaction with CHFR, several deletion mutants of SNF5 were generated (Figure 13A). FLAG-tagged wild-type SNF5 and its mutants were co-expressed with Myc-CHFR in COS-1 cells, and cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-FLAG and anti-Myc antibodies (Figure 13B). The HR3 deletion mutant of SNF5 [SNF5 (1–319)] interacted with CHFR, but the Rpt2 and HR3 deletion mutant of SNF5 [SNF5 (1–245)] did not. In addition, when the Rpt1 region was replaced by the Rpt2 region [SNF5 (1–185+Rpt2)], the deletion mutant interacted with CHFR. Taken together, these results suggest that SNF5 interacts with CHFR through the Rpt2 region of SNF5.

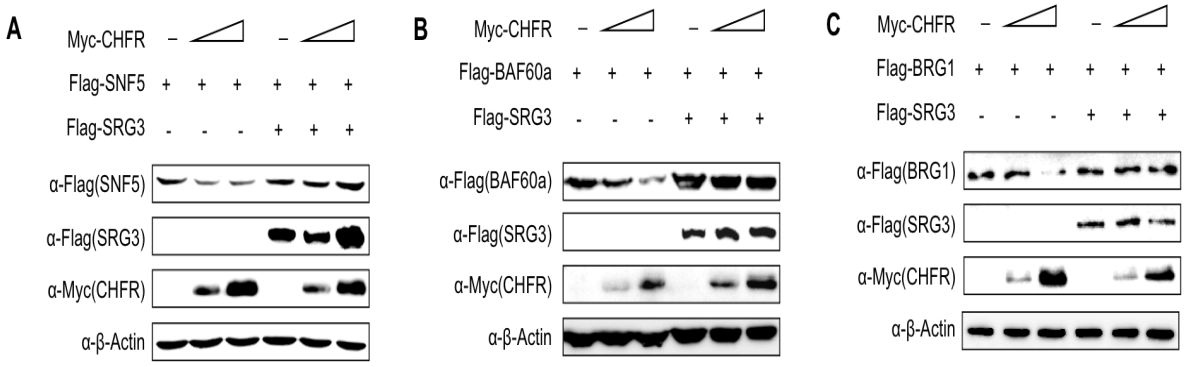


Figure 11. SRG3 can stabilize the SWI/SNF complex by interfering with CHFR.

FLAG-SNF5 (A), FLAG-BAF60a (B), or FLAG-BRG1 (C) expression vectors were co-transfected into 293T cells along with increasing amount of Myc-CHFR expression vector in the absence or presence of FLAG-SRG3 expression. After 48 hours of incubation, whole cell lysates were analyzed by immunoblot analysis with anti-FLAG and anti-Myc antibodies.

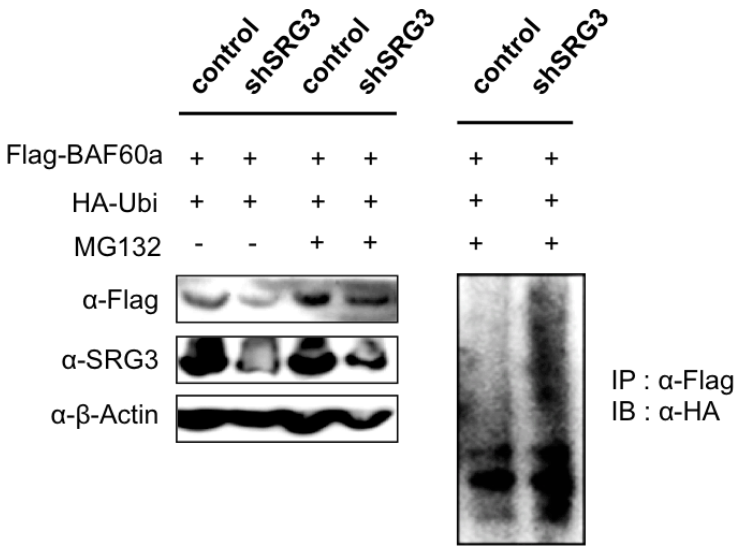
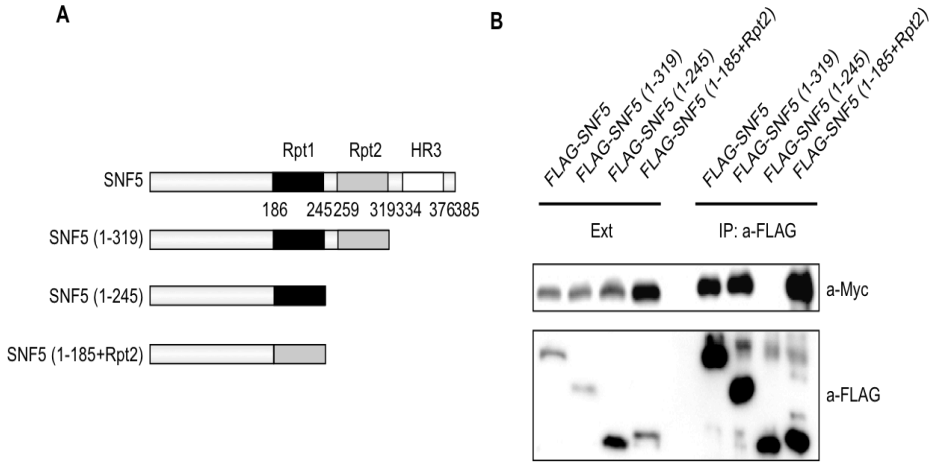


Figure 12. SRG3 stabilizes the BAF60a by inhibiting the ubiquitination of BAF60a.

NIH3T3-U6 control and NIH3T3-U6-shSRG3 stable cell lines were co-transfected with FLAG-BAF60a and HA-Ubiquitin expression vectors. After 42 hours of incubation, cells were treated with MG132 (20 μM) or vehicle for 6 hours. Whole cell lysates were immunoblotted with anti-FLAG or anti-SRG3 antibodies (left panel). Also, the cell lysates were immunoprecipitated with anti-FLAG antibody and subjected to SDS-PAGE, and immunoblotted with anti-HA antibody (right panel).

Figure 13. Rpt2 region of SNF5 is CHFR-interacting region and responsible for the degradation of SNF5 by CHFR.

(A) The schematic representation of deletion mutants of SNF5 is depicted. The Rpt1, Rpt2, and HR3 regions are indicated with solid black box, gray box, and white box, respectively. Each SNF5 mutant was tagged with FLAG epitope (not shown). (B) The interactions between deletion mutants of SNF5 and CHFR were analyzed by immunoprecipitation. COS-1 cells were co-transfected with FLAG-tagged wild type or mutant SNF5 expression vectors with Myc-CHFR expression vector. After 48 hours of incubation, whole cell lysates were immunoprecipitated with anti-FLAG antibody and subjected to SDS-PAGE, and immunoblotted with anti-Myc and anti-FLAG antibodies.


A
Rpt1
Rpt2
HR3
SNF5
186
245
259
319
334
376
385
SNF5 (1-319)
SNF5 (1-245)
SNF5 (1-185+Rpt2)
B
FLAG-SNF5
FLAG-SNF5 (1-319)
FLAG-SNF5 (1-245)
FLAG-SNF5 (1-185+Rpt2)
FLAG-SNF5
FLAG-SNF5 (1-319)
FLAG-SNF5 (1-245)
FLAG-SNF5 (1-185+Rpt2)
Ext
IP: a-FLAG
a-Myc
a-FLAG

III-2. Thymocyte-specific deletion of the deubiquitinase Bap1 impairs thymocyte development and activation

Bap1 is highly expressed in the thymus

Bap1 mRNA is expressed in several tissues, including brain, testis, placenta, and breast (Jensen et al., 1998). To investigate the Bap1 expression level in lymphoid tissues, Bap1 expression was analyzed in major immune organs, including bone marrow, thymus, spleen, and lymph node, by quantitative PCR and Western blotting. Bap1 mRNA was expressed highly in thymus and, to a lesser extent, in spleen, lymph node, and bone marrow (Figure 14A). Bap1 protein was also detectable in the thymus and other lymphoid tissues (Figure 14B). These results strongly suggest that Bap1 functions in the immune system and, especially, in the thymus. To examine this possibility in detail, *Bap1* expression was analyzed during T cell differentiation. In particular, Bap1 mRNA was highly expressed in the DN stage and decreased gradually with maturation (Figure 15A). The DN population was fractionated for further subdivision into four stages (DN1−4). *Bap1* was highly expressed in DN2 stage and was maintained through stages DN3 and DN4. Based on these observations, it was proposed that BAP1 affects early T cell differentiation and thymocyte maturation in CD4 or CD8 single-positive cells.

Impaired thymocyte development in Bap1-deficient mice

To clarify the function of Bap1 specifically in T cell development, mice bearing a LoxP-flanked gene encoding Bap1 (*Bap1*$^{fl/fl}$) were bred with mice expressing the Cre recombinase under the control of the proximal *Lck* promoter (Lck-Cre) to generate mice with Bap1-deficient thymocytes (Lck-Cre; Bap1$^{fl/fl}$). The floxed *Bap1* allele was efficiently deleted in total thymocytes from Lck-Cre; Bap1$^{fl/fl}$ mice (Figure 16). To confirm the deletion of *Bap1* during T cell development, quantitative RT-PCR was used to analyze the level of Bap1 mRNA in sorted DN cell subsets, and *Bap1* was completely deleted in DN4 stage. Next, thymocyte development was analyzed in Lck-Cre; Bap1$^{fl/fl}$ mice and control mice. The thymus gland was markedly reduced in size in Lck-Cre; Bap1$^{fl/fl}$ mice compared with control mice. The Bap1-deficient mice had about one-third fewer thymocytes than the control mice (Figure 17). Fluorescence-activated cell sorting (FACS) analysis showed that the Bap1-deficient mice had reduced percentages of CD4 SP and CD8 SP cells, but slightly increased percentages of DN cells (Figure 18A). In addition, the percentages of both peripheral CD4 SP and CD8 SP T cells in the spleen were lower, remarkably so, than in control mice (Figure 18B), and the total number of splenocytes or lymphocytes in the Bap1-deficient mice was 2- or 3-fold lower than in the control mice, respectively (Figure 17). Moreover, all sub-populations of thymocytes in

the Bap1-deficient mice had less cellularity rather than did those in the control mice (Figure 19), although the absolute number of DN cells was only mildly reduced. Furthermore, to test whether the absence of Bap1 leads to structural defects in the thymus, a histological analysis was performed. The architecture of the thymus was notably changed in the Bap1-deficient mice, with a much smaller medulla (Figure 20). Collectively, these data indicate that Bap1 is essential for normal thymocyte development.

Impaired pre-TCR signaling in Bap1-deficient DN thymocytes

As described above, Bap1 was highly expressed in DN cells, as compared to other T cell subsets (Figure 15A), and there was a smaller reduction in the ratio of DN stage cells to DP or SP cells (Figure 19). Therefore, it was questioned whether the dramatic reduction in thymic cellularity in Bap1-deficient mice could be due to an imperfection in the early T cell progenitor development during the DN stage. It was expected that Bap1 would affect thymocyte development beginning at the transition from DN3 to DN4 stage, which is a critical checkpoint for early thymic differentiation. To gain insight into the roles of Bap1 in DN cells, such cells were fractionated into DN1, DN2, DN3, and DN4 stages by staining for CD44 and CD25 as described (Ceredig and Rolink, 2002; Codfrey et al., 1993). The percentage of cells in the DN3 population was increased

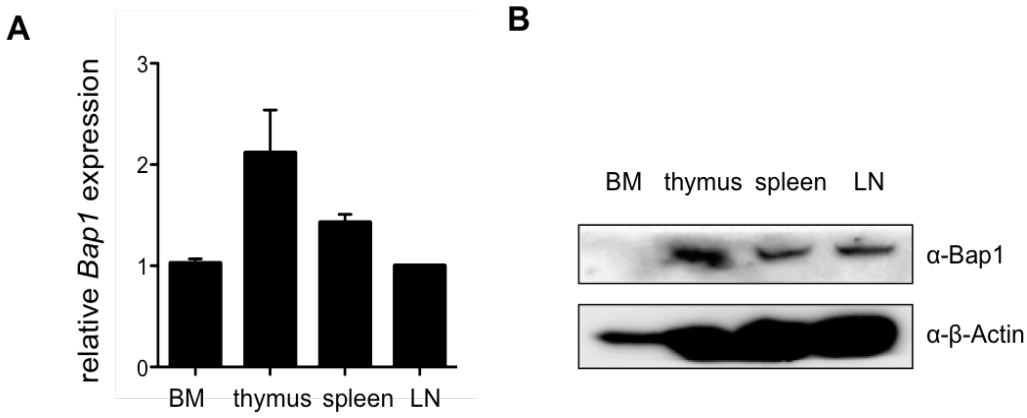


Figure 14. The expression of Bap1 mRNA and protein are high in thymus.

(A) Quantitative PCR analysis of Bap1 expression in mouse lymphoid tissues. BM, bone marrow; Thy, thymus; Spl, spleen; LN, lymph nodes. Results are presented relative to β-actin expression. Data are from two independent experiments.

(B) Immunoblot analysis of Bap1 expression in mouse lymphoid tissues. β-actin serves as a loading control. Data are representative of two independent experiments.

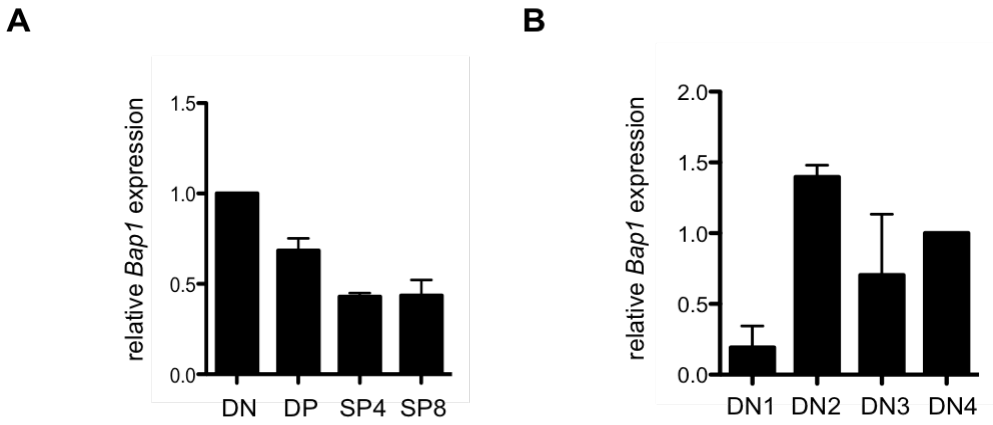


Figure 15. Bap1 mRNA is highly expressed in DN thymocyte.

(A) Quantification of Bap1 mRNA expression in sorted mouse thymocytes subsets. DN; double negative T cells; DP, double positive T cells; SP4, CD4 single positive T cells; SP8, CD8 single positive T cells. Results are presented relative to β-actin expression (n=2). (B) Quantitative PCR analysis of Bap1 mRNA expression from sorted DN1-DN4 populations. Results are presented relative to β-actin expression (n=3).

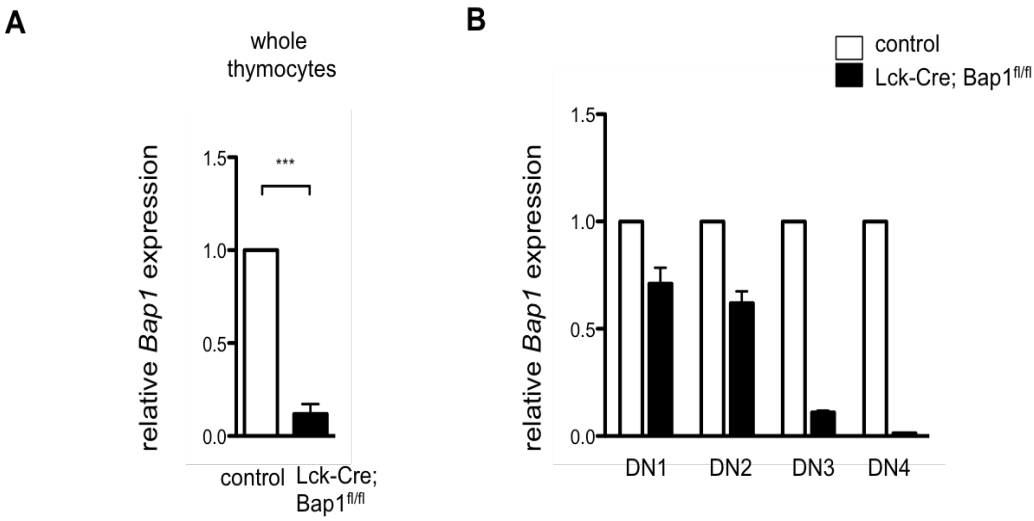


Figure 16. Deletion of the *Bap1* gene in Lck-Cre; Bap1$^{fl/fl}$ mice severely reduced the expression of Bap1 in the thymocytes.

(A) Quantitative Bap1 RT-PCR was performed on total thymocytes from the Lck-Cre; Bap1fl/fl mice and littermate control mice. Data are from four experiments. The expression of β-actin serves as a loading control. Data are shown as mean SEM.

(B) Relative expression of *Bap1* mRNA in sorted DN subsets. The expression of β-actin serves as a loading control. Data are from two experiments and shown as mean SEM.

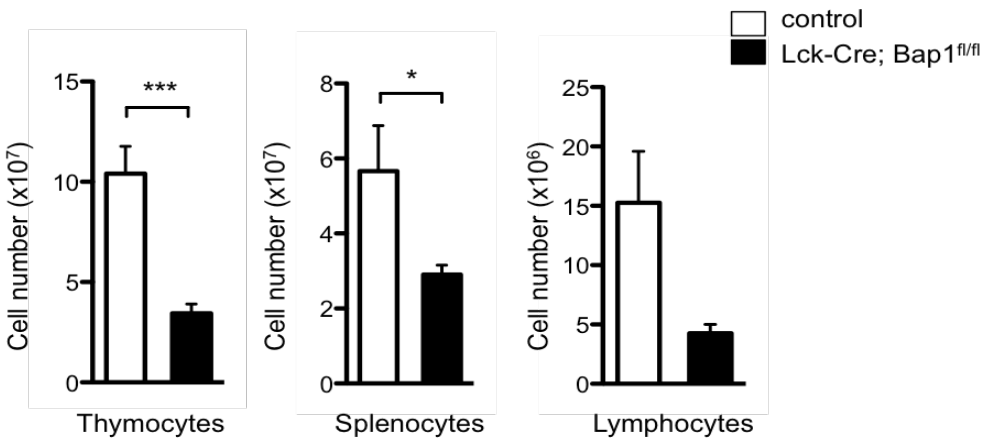


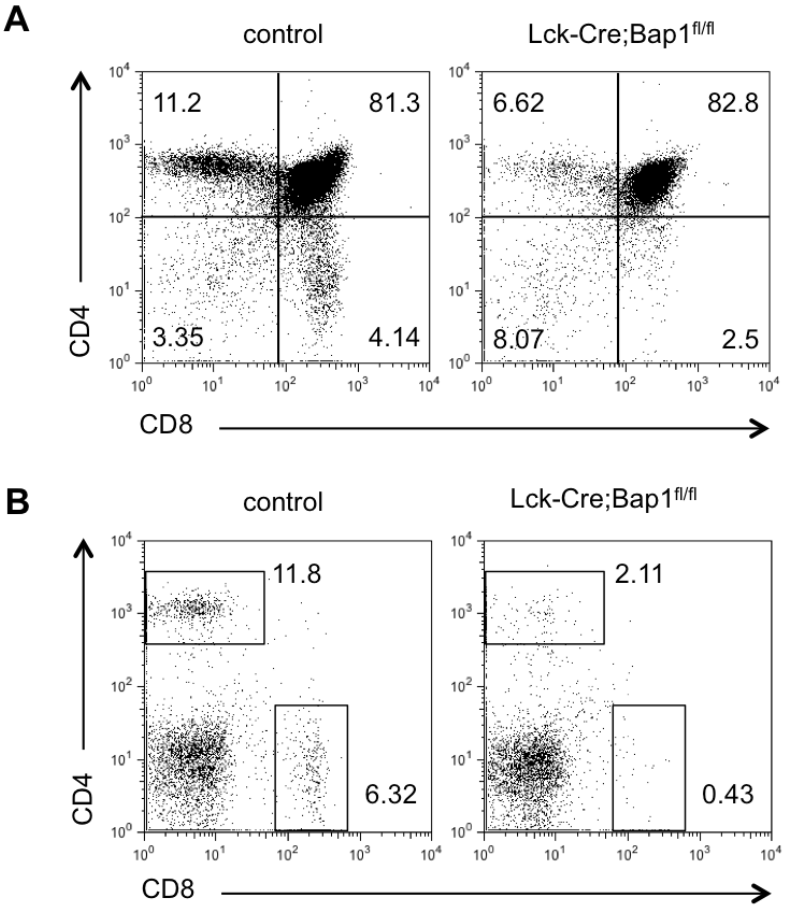
Figure 17. Thymocytes differentiation in Bap1 conditional knockout mice was impaired.

Total numbers of thymocytes (left, n=16), splenocytes (middle, n=6) and lymphocytes (right, n=2) were quantified by multiplying total live cell numbers by the fraction of cells. Error bars indicates SD.

Figure 18. T cell development is impaired in Bap1 conditional KO mice.

(A) Total thymocytes of Bap1-deficient and control mice were analyzed for CD4 and CD8 surface staining by flow cytometry. Numbers within boxes of contour diagrams indicate the percentages of cells. Data are representative of fifteen independent experiments.

(B) Phenotype of peripheral T cells was analyzed by flow cytometry by surface staining for CD4 and CD8 on control and Bap1-deficient splenocytes. Numbers adjacent to boxes of contour diagrams indicate the percentages of cells. Data are representative of six independent experiments.


A
control
Lck-Cre;Bap1fl/fl
11.2
81.3
3.35
4.14
6.62
82.8
8.07
2.5
CD4
CD8
B
control
Lck-Cre;Bap1fl/fl
11.8
6.32
2.11
0.43
CD4
CD8

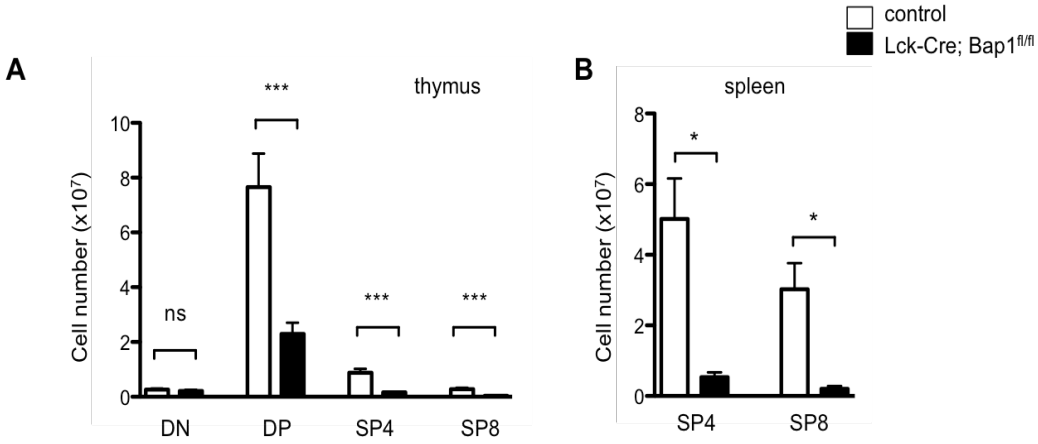
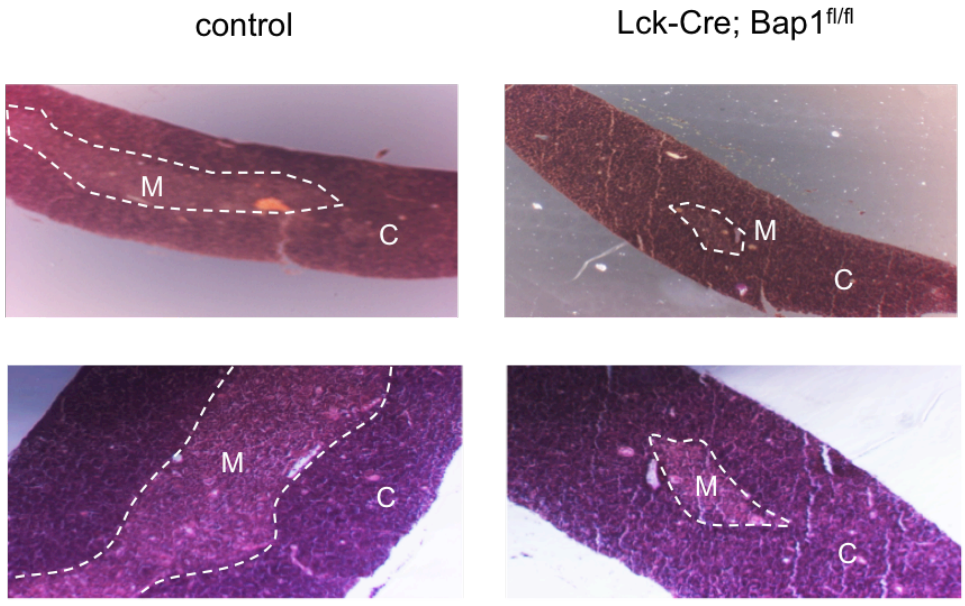


Figure 19. Bap1 deletion impacts thymic and splenic cellularity.

The absolute cell numbers of thymic subpopulations (A, n=9) or splenic subpopulations (B, n=3) in littermate control and Bap1-deficient mice. The subpopulations are determined by FACS analysis as shown in Figure 18.

Figure 20. The structure of thymus is impaired in Bap1-deficient mice.

Thymus sections from control mice and Lck-Cre; $Bap1^{fl/fl}$ mice. Each tissue section is stained with hematoxylin and eosin; the cortex (darker, outer area of dotted line) and medullar (lighter, inner area of dotted line) can be distinguished by the intensity of the staining. M, medullar; C, cortex.


control
Lck-Cre; Bap1fl/fl
M
C
M
C
M
C
M
C

among Bap1-deficient thymocytes in comparison with the controls (Figure 21A). In addition, the total cellularity of the DN3 population in Bap1 conditional knockout mice was not significantly different from that in the control mice, but it was markedly decreased in the DN4 population. Furthermore, compared to the controls, the DN3/DN4 ratio was two-fold higher in Bap1-deficient mice (Figure 21B). Taken together, these data suggest that thymocyte development in the Bap1-deficient mice, in which the floxed *Bap1* alleles were substantially deleted at DN3 stage, was arrested at DN3 stage such that the DN3-to-DN4 transition was impaired.

During the DN stage, cells committed to the T cell lineage undergo TCRβ gene rearrangements, in which the TCRβ chain associates with pre-Tα and CD3 elements to form the pre-TCR complex. This process, known as β-selection, is a critical checkpoint in T cell development (Michie and Zúñiga-pflücker, 2002). It was thought that the arrest in the DN3-to-DN4 transition in Bap1-deficient mice could be due to a defect in β-selection. To investigate this possibility, TCRβ chain expression was analyzed in DN stage cells by flow cytometry. The expression of intracellular TCRβ in the DN cell populations was reduced in Bap1-deficient mice compared with that in control mice (Figure 22A). Moreover, the surface expression of TCRβ on DN cells was greatly reduced (70−80%) in Bap1-deficient mice (Figure 22B), indicating that the block at DN3 stage might be due to a lack of sufficient TCRβ expression. In contrast, the pre-Tα transcript level in the DN cell

populations was unchanged in Bap1-deficient mice compared with control mice. Thus, I concluded that abnormal TCRβ expression was the main reason for the defect in the DN3-to-DN4 transition in Bap1-deficient mice, and that Bap1 is required for pre-TCR signaling to pass the β-selection checkpoint by providing sufficient TCRβ chain expression.

Defects in the maturation of SP thymocytes in Bap1-deficient mice

In brief, Lck-Cre; $Bap1^{fl/fl}$ mice exhibited a block in thymocyte development at DN3 stage confirming a requirement for wild-type Bap1 in pre-TCR signaling to facilitate the transition from DN to DP cells. In addition to the reduction in DP thymocyte cellularity, Bap1-deficient mice exhibited a dramatic reduction in the number of CD4 or CD8 SP cells, suggesting the existence of a defect in positive selection (Figure 19). Similar phenotypes were observed in peripheral CD4 and CD8 SP T cells in the spleen in Bap1 conditional knockout mice (Figure 19). To assess the requirement for Bap1 during positive selection in more detail, transgenic TCRs were introduced into a Bap1-deficient background. HY-specific TCR transgenic mice (RAG-2 knockout background) was introduced to the CD4-Cre; $Bap1^{fl/fl}$ mice. All thymocytes in a TCR transgenic mouse express a single TCR with the same affinity for a peptide-MHC complex. The HY TCR specifically recognizes a peptide from male HY antigen presented on $H\text{-}2D^{b}$. In female HY TCR transgenic

mice, the HY^+ thymocytes are positively selected to the CD8 lineage. The selection of T cells carrying this transgenic TCR can be followed using a clonotypic antibody, T3.70. The HY TCR transgenic Bap1-deficient female mice exhibited severely impaired production of CD8 SP T cells; the percentage of CD8 SP cells in the mutant mice was half that in the control mice (Figure 23, left panel). Additionally, staining with anti-T3.70 antibodies revealed a decreased percentage of positively selected mature CD8 SP ($T3.70^{hi}CD4^-CD8^+$) cells in Bap1-deficient HY TCR transgenic mice, as compared to relative control mice (Figure 23, middle panel). The CD4/CD8 profile of the cells gated $T3.70^{hi}$ indicated the failed maturation of CD8 SP cells (Figure 23, right panel). The absolute number of CD8 SP T cells in the Bap1-deficient mice was approximately half of that in the control mice; nevertheless no large difference in total cellularity was detected between the control background and Bap1-deficient background (Figure 24A). Thymocyte maturation occurs with down-regulation of the heat-stable antigen CD24; thus, CD24 decreases in positively selected mature SP thymocytes (Sommers et al., 2005). By staining for CD24, it was again observed fewer $CD24^{lo}$ SP T cells among the $T3.70^{hi}$ cells in CD4-Cre; $Bap1^{fl/fl}$ mice, as compared to control mice (Figure 24B). As described previously, Bap1 deficiency resulted in fewer peripheral T cells in the spleen (Figure 18B and 19). Taken together, these data demonstrate that Bap1 deficiency led to fewer mature T cells both in the thymus and in the peripheral tissues. Thus, Bap1 was dispensable for positive selection

Figure 21. The transition from DN3 to DN4 stage is blocked in Bap1-deficient mice.

(A) Single-cell suspensions of thymocytes from control or Bap1-deficient mice were stained with antibodies to CD4, CD8, CD44 and CD25. Numbers in quadrants indicate the percentage of cells. Data are representative of eight independent experiments.

(B) Left, the absolute cell numbers of thymocytes from DN1 to DN4 subpopulations (n=8). Right, ratio of DN3 T cells to DN4 T cells.

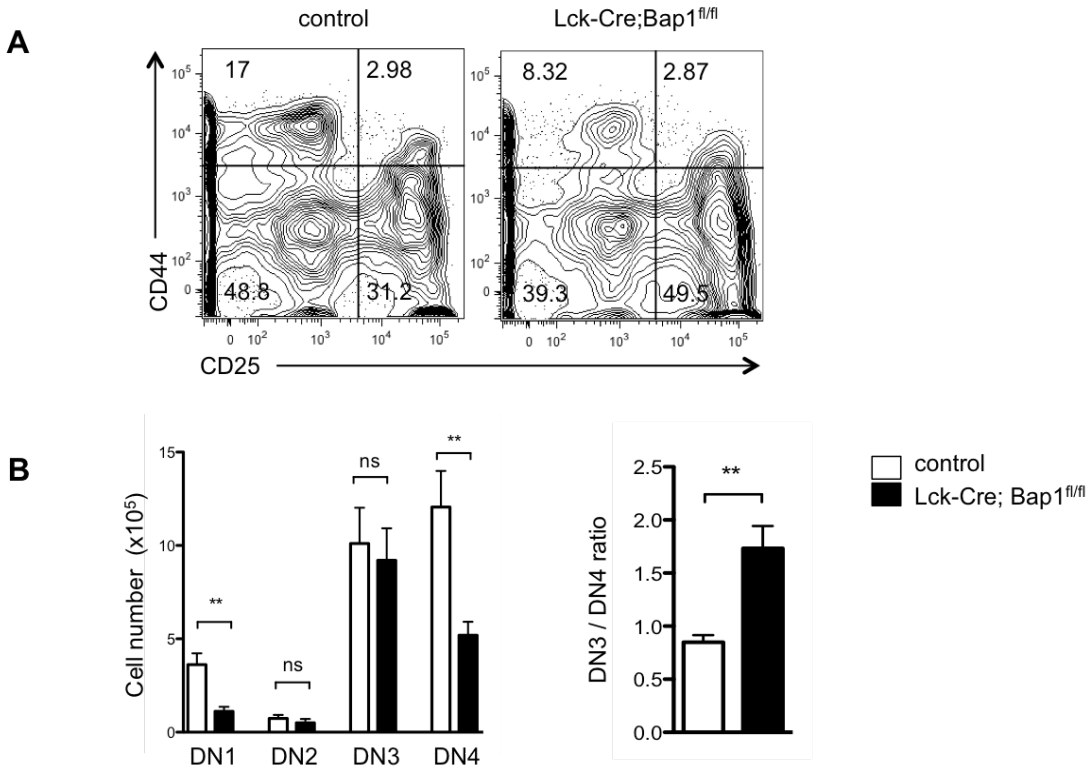
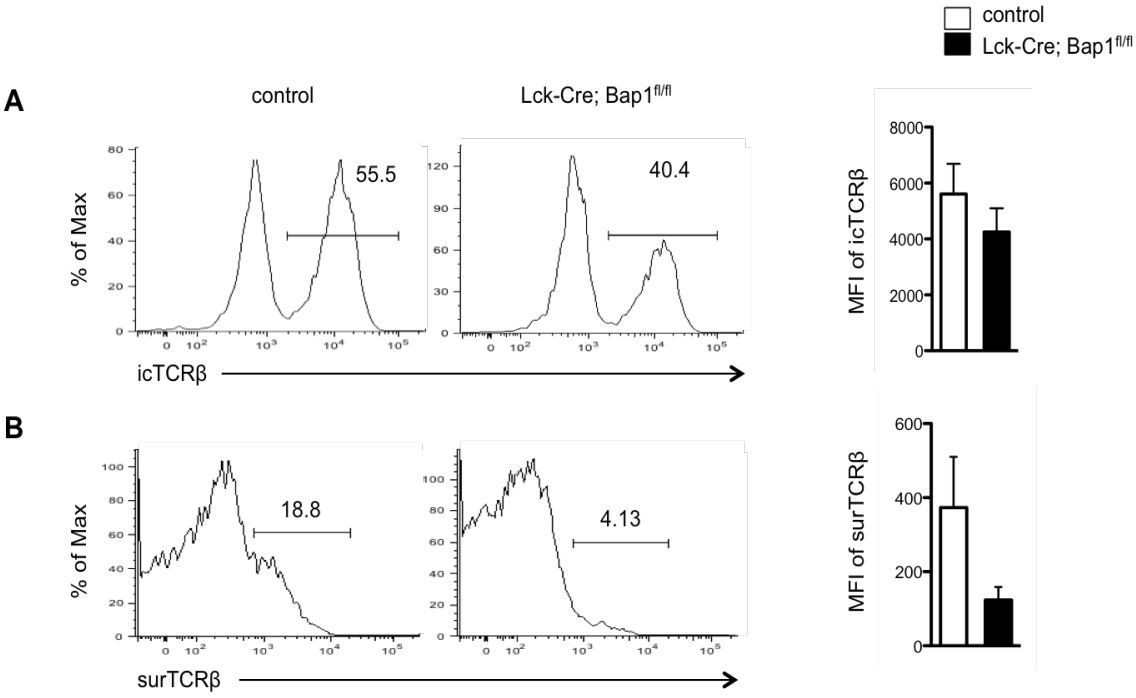

A
control
Lck-Cre;Bap1fl/fl
17
2.98
8.32
2.87
48.8
31.2
39.3
49.5
CD44
CD25
B
Cell number (x10^5)
DN1
DN2
DN3
DN4
ns
**
DN3 / DN4 ratio
control
Lck-Cre; Bap1fl/fl

Figure 22. Pre-TCR signaling is defective in Bap1-deficient mice.

(A) Intracellular staining of TCRβ on gated DN thymocytes. Numbers above bracketed lines indicate the percent of TCRβ cells. Data are representative of three independent experiments. The mean fluorescence intensity of intracellular TCRβ was calculated.

(B) Surface staining of TCRβ on gated DN thymocytes. Numbers above bracketed lines indicate the percent of TCRβ cells. Data are representative of three independent experiments. The mean fluorescence intensity of surface TCRβ was calculated.


control
Lck-Cre; Bap1fl/fl
A
control
Lck-Cre; Bap1fl/fl
% of Max
55.5
40.4
icTCRβ
MFI of icTCRβ
8000
6000
4000
2000
0
B
% of Max
18.8
4.13
surTCRβ
MFI of surTCRβ
600
400
200
0

and maturation during late T cell differentiation, as well as early T cell differentiation in the DN stage. It remains to be defined whether the positive selection of CD4 SP thymocytes is affected by a loss of Bap1, as in CD8 SP thymocytes; this issue could be resolved by crossing Bap1-deficient mice with another TCR transgenic mouse line (e.g., OT-II mice).

Weakness of TCR-mediated signaling in Bap1-deficient mice

Signaling cascades arising from the TCR drive the transition of DP thymocytes to SP thymocytes. My observation that Bap1 plays a role in the generation of SP thymocytes suggests that Bap1 contributes to TCR signal transduction in the thymus. To determine whether Bap1 affects TCR-mediated signaling, intracellular signaling events, including calcium mobilization and extracellular signal-regulated kinase (ERK) activation, were analyzed in total thymocytes from control and Bap1-deficient mice. During the positive selection of thymocytes, calcium signaling and ERK phosphorylation have pivotal roles in transducing the proximal TCR signal to downstream effectors. To analyze the requirement for Bap1 in these signaling cascades, thymocytes were activated by TCR crosslinking with anti-CD3 plus anti-CD4 antibodies or with phorbol 12-myristate 13-acetate (PMA) plus ionomycin. Thymocytes from Bap1-deficient mice exhibited a lower TCR-driven calcium flux than did thymocytes from control mice (Figure 25A). The TCR-mediated phosphorylation of ERK

by intracellular staining was also evaluated. ERK phosphorylation in Bap1-deficient mice was increased to a lesser degree than in control mice (Figure 25B). In sum, these data suggest that the loss of Bap1 resulted in the TCR-mediated activation of calcium and ERK signaling, which means that Bap1 is a critical part of TCR signaling.

Apart from thymocyte differentiation, it was also assessed whether Bap1 functions in the activation of T cells. To this end, it was assessed that the ability of Bap1-deficient sorted naive splenic CD4 SP or CD8 SP T cells ($CD44^{lo}CD62L^{hi}CD4^{+}$ or $CD44^{lo}CD62L^{hi}$ $CD8^{+}$, respectively) to respond to stimulation with plate-bound anti-CD3 and anti-CD28 antibodies. Unexpectedly, a greater percent of memory T cells ($CD44^{hi}CD62L^{lo}$) was increased in Lck-Cre; $Bap1^{fl/fl}$ mice, as compared to control mice (Figure 26A). This could be explained by the fact that the absolute numbers of naive and memory CD4 or CD8 SP T cells were significantly downregulated in the Bap1-deficient mice (Figure 26B). When sorted naive T cells were cultured in medium only, the T cell activation marker CD69 was unchanged in both control and Bap1-deficient mice (Figure 26C). However, upon the activation of naive T cells via the TCR with anti-CD3 and -CD28 antibodies, CD69 expression was induced on the surface of CD4 or CD8 SP splenic T cells in the control mice. The SP T cells of the Bap1-deficient mice also showed an increased CD69 expression level, but the levels were lower than in control mice. This observation indicates that the loss of Bap1 led to a reduction in

the efficiency of TCR signaling. Thus, Bap1 contributes to TCR-associated responses for T cell activation.

Figure 23. The positive selection of CD8 SP cells is impaired in Bap1-deficient mice.

(Left) Flow cytometry of thymocytes from control or Bap1-deficient female mice expressing a transgene encoding the MHC class I−restricted HY TCR. Staining of CD4 and CD8 on total thymocytes. Numbers adjacent to boxes of contour diagrams indicate the percentage of cells.
(Middle) Staining with antibody to the HY-specific antibody T3.70. Numbers above bracketed lines indicate percent T3.70-positive cells.
(Right) Flow cytometry profiles of the expression of CD4 and CD8 on gated T3.70^{high} thymocytes in Bap1-deficient mice or control mice.
Data are representative of three independent experiments.

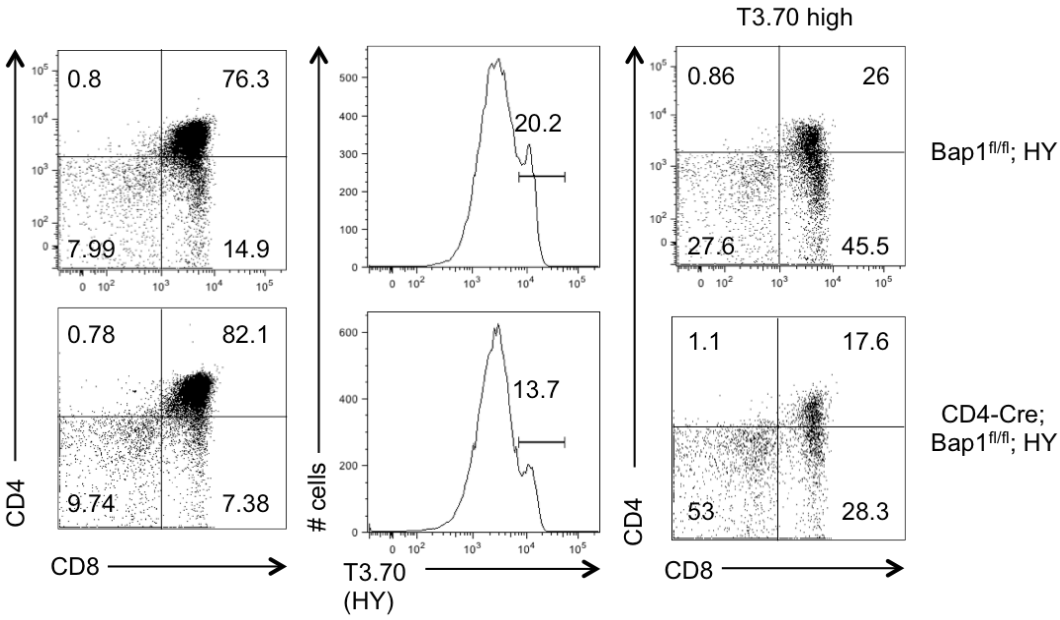

T3.70 high
0.8
76.3
7.99
14.9
0.78
82.1
9.74
7.38
CD4
CD8
cells
20.2
13.7
T3.70
(HY)
0.86
26
27.6
45.5
1.1
17.6
53
28.3
Bap1fl/fl; HY
CD4-Cre;
Bap1fl/fl; HY

Figure 24. The maturation of thymocytes is defected in Bap1-deficient mice.

(A) Total cell number of thymocytes (left, n=3), splenocytes (middle, n=2) and thymic CD8 T cells (right, n=3) of CD4-Cre; $Bap1^{fl/fl}$; HY mice and $Bap1^{fl/fl}$; HY mice were quantified. Data are shown as mean SEM.

(B) FACS analysis by staining of CD24 and T3.70 on total thymocytes of CD4-Cre; $Bap1^{fl/fl}$; HY mice and $Bap1^{fl/fl}$; HY mice. Numbers adjacent to boxes of contour diagrams indicate the percentage of cells. Data are representative of three independent experiments.

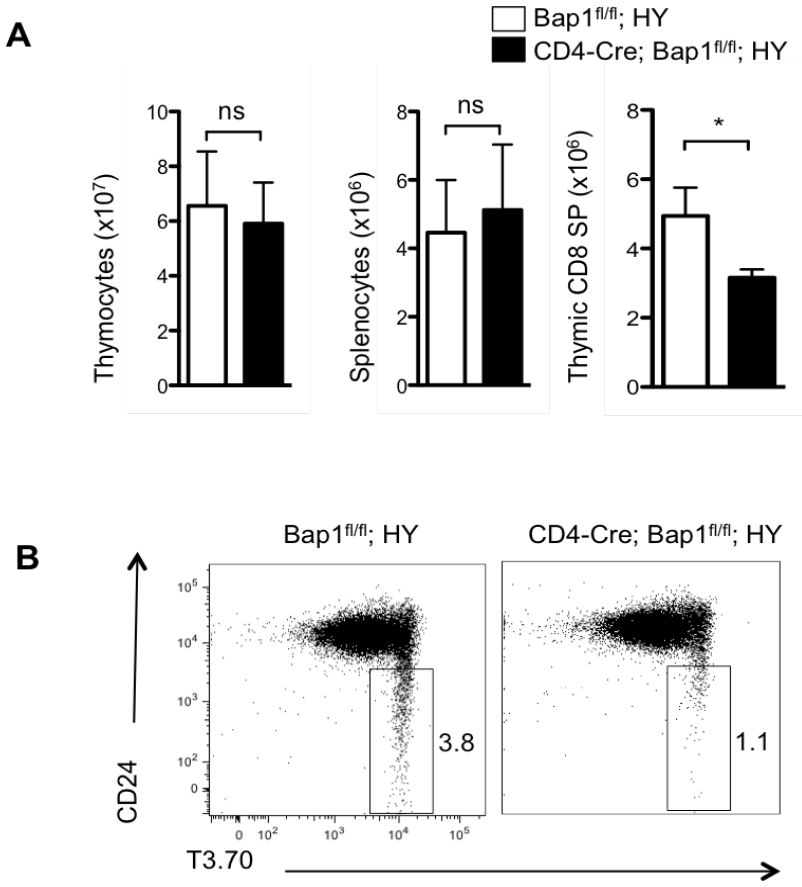

A
Bap1fl/fl; HY
CD4-Cre; Bap1fl/fl; HY
Thymocytes (x10^7)
Splenocytes (x10^6)
Thymic CD8 SP (x10^6)
ns
ns
*
B
Bap1fl/fl; HY
CD4-Cre; Bap1fl/fl; HY
CD24
T3.70
3.8
1.1

Figure 25. TCR signaling is impaired in Bap1 conditional KO mice.

(A) Calcium flux in total thymocytes from control and Lck-Cre; $Bap1^{fl/fl}$ mice after stimulation with anti-CD3-biotin antibody and anti-CD4-biotin antibody, followed by streptavidin crosslinking. The downward arrow indicates the time when streptavidin was added. Data are representative of three independent experiments.

(B) ERK phosphorylation of total thymocytes from Lck-Cre; $Bap1^{fl/fl}$ mice and littermate control mice by TCR stimulation was analyzed by intracellular staining.

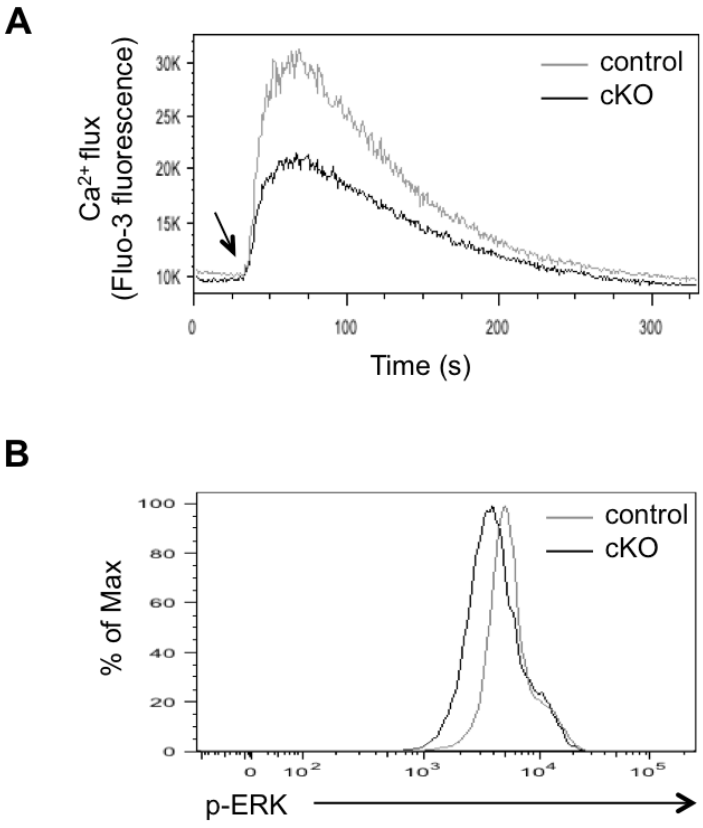
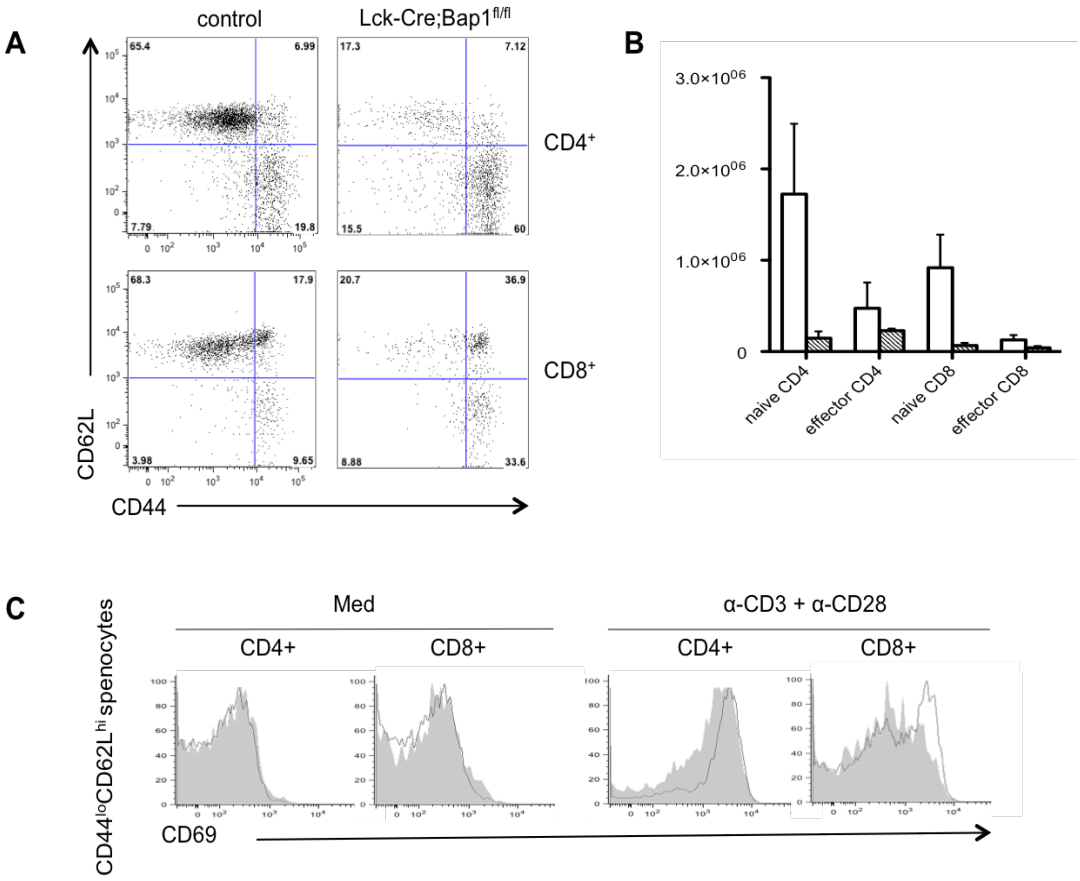

A
Ca2+ flux
(Fluo-3 fluorescence)
30K
25K
20K
15K
10K
0
100
200
300
Time (s)
control
cKO
B
% of Max
100
80
60
40
20
0
0
10^2
10^3
10^4
10^5
p-ERK
control
cKO

Figure 26. Deficiency of Bap1 down-regulates the activation of peripheral T cells.

(A) FACS analysis of the expression of CD44 and CD62L on gated $CD4^+$ or $CD8^+$ splenocytes from control and Bap1-deficient mice. Numbers in quadrants indicate the percentage of cells. Data are representative of three independent experiments.

(B) The absolute cell numbers of $CD44^{lo}CD62L^{hi}$ naive cells and $CD44^{hi}CD62L^{lo}$ memory cells were calculated from three independent experiments. Data are shown as mean SEM.

(C) Flow cytometry of CD69 staining on gated $CD4^+$ or $CD8^+$ T cells from control (solid line) or Lck-Cre; $Bap1^{fl/fl}$ (shaded histogram) sorted $CD44^{lo}CD62L^{hi}$ splenocytes left unstimulated (Med) or stimulated by crosslinking with anti-CD3 and anti-CD28 for 5 hours. Data are representative of two independent experiments.


A
control
Lck-Cre;Bap1fl/fl
CD4+
CD8+
CD62L
CD44
65.4
6.99
7.79
19.8
17.3
7.12
15.5
60
68.3
17.9
3.98
9.65
20.7
36.9
8.88
33.6
B
3.0×10^06
2.0×10^06
1.0×10^06
0
naive CD4
effector CD4
naive CD8
effector CD8
C
Med
α-CD3 + α-CD28
CD4+
CD8+
CD4+
CD8+
CD44loCD62Lhi spenocytes
CD69

CHAPTER IV.

DISCUSSION

IV-1. SRG3 stabilizes the SWI/SNF complex by blocking CHFR mediated ubiquitylation and degradation

Post-translational modification of proteins by ubiquitin ligase is a central regulator in a variety of cellular processes. The SWI/SNF complex is a major transcriptional regulator, and therefore it needs to be carefully regulated. SRG3/mBAF155 has been found to play a role as a scaffold for other components of the SWI/SNF complex (Sohn et al., 2007), enabling the complex to function efficiently when required. It was demonstrated that the major components of the complex BRG1, SNF5, and BAF60a were stabilized by ectopic expression of SRG3 *in vitro*. Furthermore, BAF155, a human homolog of SRG3, has been reported to mediate the stabilization of BAF57 expression (Keppler and Archer, 2010). From results in this paper, it was concluded that BRG1, SNF5, and BAF60a were ubiquitylated and degraded via the 26S proteasome-mediated pathway (Sohn et al., 2007). However, the identity of the ubiquitin ligases involved in this process has been unknown. Recent studies have shown that E3 ubiquitin ligases are important for the regulation of the SWI/SNF complex. TRIP12 interacts with BAF57 and ubiquitinates it (Keppler and Archer, 2010). Unkempt, another ubiquitin ligase, is involved in BAF60b ubiquitylation in a Rac1-dependent manner, which increases the degradation of BAF60b but not of BAF60a or BAF60c (Lorès et al.,

2010). Here, I show that CHFR, an E3 ubiquitin ligase, ubiquitinates and directs BRG1, SNF5, and BAF60a to a proteosomal degradation pathway.

CHFR was found to associate with several chromatin remodeling factors, which control chromosome stability, and to function as a tumor suppressor. CHFR ubiquitinates and negatively regulates histone deacetylase 1 (HDAC1), promoting *p21* gene expression to induce *p21*-dependent cell cycle arrest (Oh et al., 2009). It also synergistically maintains genomic stability with another E3 ubiquitin ligase, ring finger protein 8 (RNF8), and inhibits tumorigenesis by modulation of histone modifications and suppression of ataxia telangiectasia mutated (ATM) kinase activation (Wu et al., 2011). CHFR and RNF8 double-knockout mice showed low H2A and H2B ubiquitylation, H4K16 acetylation in thymocytes and suppressed ATM activation in response to DNA damage response, thereby causing T-cell lymphoma to develop. CHFR functions as a regulator controlling the stability of HLTF, which belongs to the SWI/SNF chromatin remodeling complex family (Kim et al., 2010). All these results indicate that CHFR is involved in the regulation of chromatin structure by modulating histone modifications and/or ubiquitinating several different chromatin remodeling proteins.

The SWI/SNF complex has been shown to have the activity of a tumor suppressor. It physically interacts with Rb and mediates Rb-mediated cell cycle arrest (Dunaief et al., 1994). In addition, SNF5 represses cyclin D1 expression by recruiting the HDAC complex to its

promoter (Tsikitis et al., 2005; Zhang et al., 2002). The SWI/SNF complex also associates with BRCA1 or c-Myc (Bochar et al., 2000; Roberts and Orkin, 2004) and controls p53-mediated transcriptional activity, which regulates cell cycle arrest (Ahn et al., 2011; Levine, 1997). Some studies have shown that inactivation of SNF5 result in a failure of cell cycle arrest caused by p53 defects (DelBove et al., 2009; Isakoff et al., 2005). SRG3 was also shown to function as a tumor suppressor by modulating $p21^{WAF1/CIP1}$ expression (Ahn et al., 2011).

In contrast, other studies have shown that the expression of the SWI/SNF subunits is maintained in human tumor cells. The increase in BRG1 level is associated with enhanced tumor cell growth and invasion in human gastric and prostate cancer (Sentani et al., 2001; Sun et al., 2007). BAF57 activity is maintained in prostate cancer while supporting androgen receptor (AR) activity; BAF57 inhibitory peptide sufficiently blocks androgen-dependent prostate cancer cell proliferation in AR-positive cells (Link et al., 2008; Link et al., 2005). These results make it clear that the robust expression of subunits of the SWI/SNF complex can induce cancer cell growth and invasion in a cell context-dependent manner. The pathway involved in the maintenance of expression of the SWI/SNF complex in several cancer cells remains unknown. Therefore, it is crucial to find the mechanism by which the protein level of the SWI/SNF complex is regulated. Thus, it is notable that CHFR, a tumor suppressor, regulates the ubiquitylation and degradation of the SWI/SNF

chromatin remodeling proteins. It is conceivable that CHFR functions as a tumor suppressor also by reducing the expression level of the components of the SWI/SNF complex in a context-dependent manner with implications for the understanding of epigenetics of cell cycle control and potentially, cancer pathogenesis. This hypothesis needs to be investigated further.

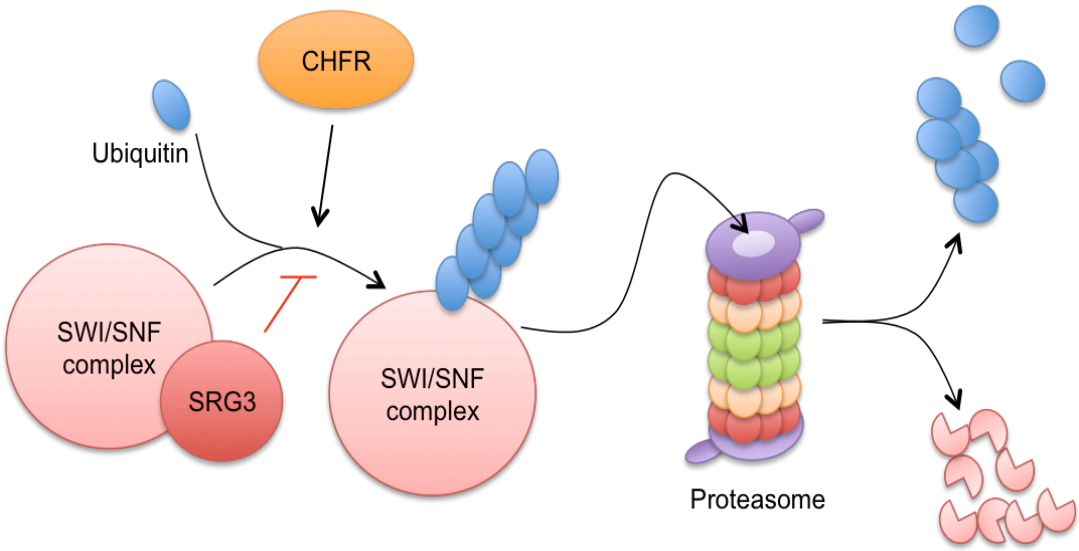


Figure 27. The stabilization model of the SWI/SNF complex.

IV-2. Thymocyte-specific deletion of the deubiquitinase Bap1 impairs thymocyte development and activation

The significance of Bap1 in the regulation of thymocyte development and activation has not been defined, although previous reports have demonstrated the defects of Bap1 led to an aberrant developmental phenotypes in hematopoietic cells (Abdel-Wahab et al., 2012; Abdel-Wahab and Dey, 2013; Peña-Llopis et al., 2012). To elucidate the roles of Bap1 in thymus, I used mice for loxP-flanked *Bap1* alleles deleted by T-cell specific Lck promoter-driven Cre recombinase that therefore lack Bap1 specifically in T cells. After generation of Lck promoter-driven Bap1 conditional knockout mice, it was confirmed that highly expressed mRNA and protein of Bap1 in thymus were efficiently deleted from Lck-Cre; $Bap1^{fl/fl}$ mice by quantitative real time PCR and western blotting, respectively. The thymus size and total thymocyte cellularity were significantly reduced in Bap1-deficient mice compared to littermate control mice. It was very interesting because Bap1 is usually known as a tumor suppressor from many researches. So it was expected that Bap1 would have a different role in thymocytes from other hematopoietic lineage cells and suspected that Bap1 could affect the cell death that would lead to the marked decrease in the thymic cellularity when Bap1 was lost. To confirm that, total thymocytes were stained with Annexin V and propidium iodide and analyzed by flow cytometry.

Unexpectedly, there was no significant difference in the thymocytes profile of Annexin V/propidium iodide between Bap1-deficient mice and littermate control mice. To analyze the proliferation of Bap1-deficient thymocytes, BrdU was injected i.p. and mice were sacrificed 4 hour later before BrdU incorporation was measured. The cell proliferation was also not changed with Bap1 deletion in all thymic subpopulations. It is suggested the possibility that, in thymus, Bap1 is involved in cellular processes other than apoptosis or proliferation to regulate thymic development.

From this paper, it was shown that reduced expression of TCRβ chain of the thymocytes and developmental block at DN3 stage in Bap1-deficient mice. During thymocyte differentiation, only thymocytes that have generated a functional TCRβ chain become selected to differentiate to the $CD4^{+}CD8^{+}$ double positive stage. Dβ-Jβ rearrangement is initiated at both alleles of the TCRβ gene loci, followed by Vβ-DβJβ rearrangement at individual alleles. The TCRβ chain covalently couples to the non-polymorphic pre-Tα chain, which together with the CD3 molecules form the pre-TCR complex. Bap1-loss resulted in developmental block at DN3 stage, which suggested a requirement of Bap1 for the DN3-DN4 transition. Furthermore, results obtained from this paper showed that the percentages and the absolute cell numbers of CD4 and CD8 SP cells were significantly decreased with Bap1-loss. In the attempt to assess the effect of Bap1 defect for the CD4/CD8 SP

maturation, the CD4-Cre; Bap1$^{fl/fl}$ mice were crossed with HY-TCR transgenic mice. HY-TCR transgenic mice are useful to investigate the positive selection or negative selection in female mice or male mice, respectively. It was observed that the maturation of CD8 SP thymocytes was impaired in HY TCR transgenic Bap1-deficient female mice. These outcomes identified Bap1 as an important factor for the positive selection of CD4/CD8 SP T cells. There was no difference in male mice with or without Bap1, which suggests that Bap1 does not affect negative selection of thymocytes. Further work is also needed to elucidate whether Bap1 actually influences the positive selection of CD4 SP cells as well as CD8 SP cells.

The Ras-ERK and the calcium signaling pathways are required for the positive selection of thymocytes and the activation of peripheral T cells. In order to closely observe the TCR signaling, calcium influx and phosphorylated ERK level in total thymocytes were investigated after stimulation with anti-CD3 or PMA and ionomycin. It was found that the calcium flux and ERK activation were downregulated in Bap1-deficient mice compared to the control mice, suggesting that loss of Bap1 led to the insufficient TCR signal transduction. And with stimulation *in vitro*, the naive splenic T cells were not efficiently activated, which is represented by down-modulated CD69 expression level, in Bap1-deficient mice compared to control mice. Thus, T cell-specific deletion of Bap1 resulted in a weakness of TCR-mediated signal followed by inefficiently activated

T cells. From all these results, I conclude that Bap1 serves as a critical factor during thymocytes development through the β-selection at DN stage, positive selection of SP cells and activation of peripheral T cells.

One mechanism by which Bap1 could influence the development and activation of T cells would be through the chromatin remodeling by ubiquitination of histone as a PR-DUB complex to bind at target genes and to remove mono-ubiquitin from histone H2AK119 (Abdel-Wahab and Dey, 2013; Harbour et al., 2010; Scheuermann et al., 2010; Wiesner et al., 2011). The mutant mice of Asxl1, another core subunit of PR-DUB complex, showed a dramatic reduction in total thymocytes cellularity compared with control mice (Fisher et al., 2010) similarly to Bap1-deficient mice. The CD4/CD8 subpopulations also showed a significant decrease in absolute cell numbers in the Asxl1 mutant mice. Although the defective phenotypes of thymocytes development in mutant mice looked generally similar, there were some differences between Bap1 and Asxl1 mutant mice. The results from Asxl1 mutant mice were observed only in mice older than 15 weeks not in younger mice and there were no markedly differences in the absolute cell numbers of peripheral CD4/CD8 SP cells or DP cells in Asxl1 mutant mice unlikely in Bap1-deficient mice. Previous studies also have suggested the possibility that Bap1 and ASXL1 may work independently (Abdel-Wahab and Dey, 2013; Dey et al., 2012). Thus, I suggest that Bap1 and Asxl1 may function independently during thymocytes development, although there are some differences such as a

deletion system that the mutant mice of Asxl1 are not designed to control the onset timing of ablate Asxl1 in thymus using a conditional mice.

Proteins interacting with Bap1 have been identified and the representative ones are HCF-1 (Host cell factor 1) and OGT (O-linked β-N-acetyl-glucosamine transferase) (Dey et al., 2012; Machida et al., 2009; Misaghi et al., 2009; Yu et al., 2010; Yu et al., 2014). HCF-1 is an important transcriptional regulator of the cell cycle, which associates with diverse histone-modifying molecules such as histone methyltransferases. Bap1 KO splenocytes showed decreased HCF-1 expression and Bap1 deubiquitinases HCF-1 to inhibit its proteasomal degradation (Dey et al., 2012). Also, Bap1 form a complex with OGT and regulates the stability of OGT. OGT is an enzyme involved the O-GlcNAc (O-linked β-N-acetyl-glucosamine) cycling, which catalyzes the addition of the O-GlcNAc to proteins (Mcclain et al., 2002). In addition to that Bap1 directly regulates the stability of HCF-1 or OGT, it is possible that those molecules form a complex to affect the stability or function of another protein such as peroxisome proliferator activator receptor γ coactivator 1α (PGC-1α) (Yang et al., 2012). Bap1 deubiquitylates and thus stabilizes OGT, which O-GlcNacylates HCF1 and activates it. Activated HCF1 recruits OGT to O-GlcNacylate PGC-1α. This modification enables Bap1 to bind and deubiquitylate PGC-1α that is stabilized and can promote gluconeogenesis. Bap1 has also been shown to form a ternary complex with HCF1 and the transcription factor Yin Yang 1 (YY1) that controls

the transcription of genes involved in cell proliferation. Specifically, Bap1 interacts with the zinc fingers of YY1 through its coiled-coil motif and is recruited together with HCF1 to the promoter of the gene that encodes a component of the mitochondrial respiratory chain3 (Yu et al., 2010). Immunoprecipitation assay in mouse showed Bap1 interacted with HCF-1 and OGT in the spleen (Dey et al., 2012). Using a proteomic approach, it was identified that HCF-1 was an interacting protein of Bap1 also in thymus. Furthermore, previous study using T cell-specific mutagenesis of the *Ogt* allele presented the necessity of the OGT in the thymic development (O'Donnell, 2004) and several evidences have suggested the relation between glycosylation and the thymocyte development (Gambetta et al., 2009; Marth and Grewal, 2008; Zhou et al., 2014). Further work is needed to elucidate how exactly Bap1 and other molecules form a complex in thymus and what the target genes are to be affected by the complex. In this paper, I propose that Bap1 roles as a critical regulator with variable partners to successfully complete the thymocyte development, maturation and activation.

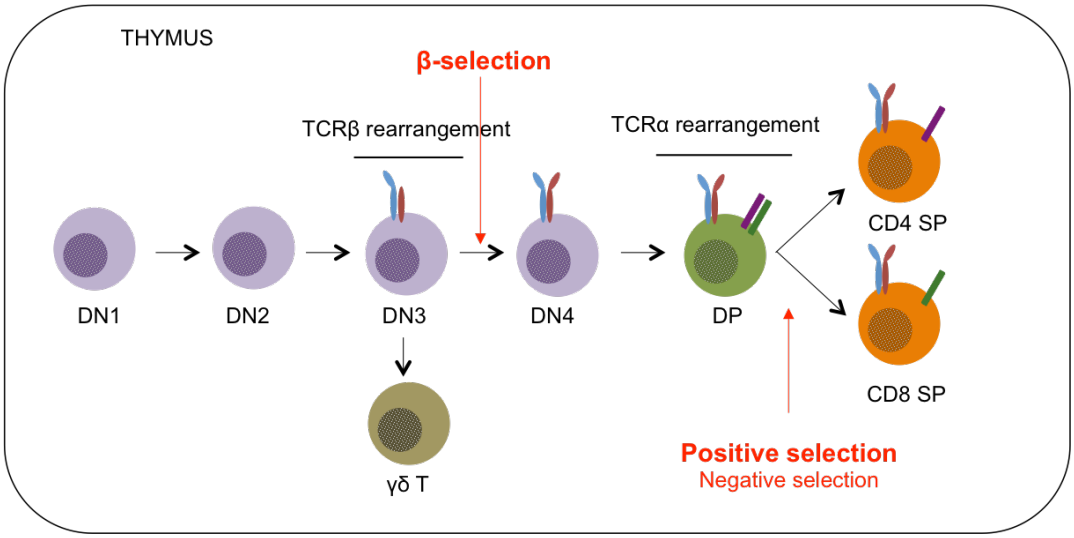


Figure 28. The model for the function of Bap1 in the thymocyte development.

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국문 초록

SRG3에 의한 SWI/SNF 염색사 리모델링 복합체의 안정화 및 BAP1 이 흉선세포 분화에 미치는 영향에 대한 연구

정 인 경

T 세포는 세포성 면역 반응에 있어서 중요한 매개체이다. 골수에서 유래한 T 세포 전구체는 흉선으로 들어가 체계적이고 복잡한 과정을 거쳐 분화, 선택 과정을 지 기능을 가지는 T 세포로 성숙한다. T 세포 분화가 일어나는 단계들은 계통 특이적 유전자의 전사 조절과 TCR 유전자의 재배열에 의해 제어된다. Notch, GATA-3, E2A/HEB, Id, c-Myb, ThPOK, Runx 복합체, Ikaros 등의 다양한 유전자의 전사 유무가 T 세포의 계통 결정에 중요한 역할을 한다. 또한 TCR 유전자의 재배열이 성공적으로 일어나야만 통과할 수 있는 β-selection 과 긍정적 선별과정 역시 성숙한 T 세포의 생성에 필수적이다. 히스톤 수정 효소와 염색사 리모델링 복합체에 의해 조절되는 염색사의 상태가 유전자의 발현 여부를 결정한다.

이 논문에서 우리는 염색사의 상태를 변화시키는 역할을 하는 염색사 리모델링 복합체인 SWI/SNF 복합체의 주요 구성

단백질에 대해 연구하였다. BRG1, SNF5, BAF60a 가 유비퀴틴화 되어 분해되는 과정을 SRG3 가 억제한다는 것이 이전 연구를 통해 알 수 있었다. 선행연구를 통해 SWI/SNF 염색사 리모델링 복합체가 흉선세포의 분화과정에 중요한 역할을 한다는 것과 SWI/SNF 복합체의 안정화는 유비퀴틴화에 의해 조절된다는 것을 알 수 있었다. 이 논문에서는 특별히 SRG3 가 SNF5, BRG1, BAF60a 의 프로테아솜에 의해 매개되는 분해 과정을 억제함으로써 그 단백질들을 안정화시켜 궁극적으로 SWI/SNF 복합체의 안정화에 중요한 역할을 한다는 것을 밝혀내었다. 한편, E3 유비퀴틴 결합효소인 CHFR 은 종양 억제 인자로 잘 알려져 있으며 세포주기 조절과 종양 생성과정에 중요한 역할을 한다. CHFR 은 BRG1, SNF5, BAF60a 에 결합하여 유비퀴틴화 시킴으로써 프로테아솜 매개 과정을 통해 분해 되도록 유도한다. SRG3 는 이러한 CHFR 과 다른 분자들의 상호작용을 억제함으로써 복합체의 안정화를 가져온다는 사실을 밝혀내었다.

다음으로 우리는 탈유비퀴틴효소인 Bap1 의 흉선세포 분화 과정에서의 기능에 대해 알아보았다. Bap1 결핍 생쥐는 배아상태에서의 발생 이상과 미엘로이드 계통 세포 증식 등의 비정상적인 현상을 보인다. 이 연구에서는, Bap1 이 T 세포 분화에 미치는 영향을 알아보기 위하여 Lck-Cre mouse 를 이용하여 Bap1

유전자를 흉선에서 특이적으로 제거하였다. 그를 통하여 pre-TCR 신호전달 과정과 TCR 신호 전달을 통한 T 세포 분화와 활성화에 Bap1 이 중요한 역할을 한다는 것을 밝혀내었다. Bap1 결핍 T 세포는 DN3 에서 DN4 단계로 넘어가지 못하며 TCRβ 발현이 낮아진다. 또한 TCR 형질 도입 생쥐 (HY-TCR 형질 도입 생쥐)에서 Bap1 이 결핍되는 경우, T 세포의 긍정적 선별과정 역시 현저히 감소되는 것을 확인할 수 있다. TCR 을 통해 들어오는 신호에 의해 발생하는 칼슘 증가와 ERK 의 활성화 역시 Bap1 이 결핍되는 경우 제대로 일어나지 않으며, TCR 자극에 의한 말초 T 세포의 활성화에도 문제가 발생한다. 이 결과들을 통하여, Bap1 이 T 세포 발생에 중요한 인자임을 확인하였으며 흉선세포의 활성화에 필요한 TCR 신호 전달 과정에도 Bap1 이 관여한다는 것을 밝혀내었다.

주요어: T 세포 분화, SWI/SNF 복합체, SRG3, CHFR, Bap1, 유비퀴틴화

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