

The Texas Medical Center Library

DigitalCommons@TMC

The University of Texas MD Anderson Cancer  
Center UTHealth Graduate School of  
Biomedical Sciences Dissertations and Theses  
(Open Access)

The University of Texas MD Anderson Cancer  
Center UTHealth Graduate School of  
Biomedical Sciences

8-2011

## SUMO-SPECIFIC PROTEASE 1 CONTROLS LYMPHOID DEVELOPMENT THROUGH REGULATION OF SUMOYLATION/ ACETYLTATION SWITCH IN STAT5

Thang Van Nguyen

Follow this and additional works at: [https://digitalcommons.library.tmc.edu/utgsbs\\_dissertations](https://digitalcommons.library.tmc.edu/utgsbs_dissertations)



Part of the [Life Sciences Commons](#), and the [Medicine and Health Sciences Commons](#)

### Recommended Citation

Nguyen, Thang Van, "SUMO-SPECIFIC PROTEASE 1 CONTROLS LYMPHOID DEVELOPMENT THROUGH REGULATION OF SUMOYLATION/ACETYLTATION SWITCH IN STAT5" (2011). *The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access)*. 162.

[https://digitalcommons.library.tmc.edu/utgsbs\\_dissertations/162](https://digitalcommons.library.tmc.edu/utgsbs_dissertations/162)

This Dissertation (PhD) is brought to you for free and open access by the The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences at DigitalCommons@TMC. It has been accepted for inclusion in The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact [digitalcommons@library.tmc.edu](mailto:digitalcommons@library.tmc.edu).

The  
**TMC LIBRARY**  
Health Sciences Resource Center

**SUMO-SPECIFIC PROTEASE 1 CONTROLS  
LYMPHOID DEVELOPMENT THROUGH REGULATION OF  
SUMOYLATION/ACETYLTATION SWITCH IN STAT5**

**by**

**Thang Van Nguyen, D.V.M., M.S.**

**APPROVED**

---

**Edward T.H. Yeh, M.D.  
(Supervisory Professor)**

---

**Chen Dong, Ph.D.**

---

**Shao-Cong Sun, Ph.D.**

---

**Phillip Carpenter, Ph.D.**

---

**Sue-Hwa Lin, Ph.D.**

**APPROVED**

---

**Dean, The University of Texas  
Graduate School of Biomedical Sciences at Houston**

**SUMO-SPECIFIC PROTEASE 1 CONTROLS  
LYMPHOID DEVELOPMENT THROUGH REGULATION OF  
SUMOYLATION/ACETYLATION SWITCH IN STAT5**

**A**

**DISSERTATION**

**Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
M. D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment of the Requirements**

**for the Degree of**

**DOCTOR OF PHILOSOPHY**

**By**

**Thang Van Nguyen, D.V.M., M.S.**

**Houston, Texas**

**August 2011**

## **DEDICATION**

This dissertation is dedicated to my parents, my beloved wife, Nhan Vu, and my lovely sons, Bach Nguyen and David-Minh Nguyen for their love, constant encouragement, support and patience.

## **ACKNOWLEDGEMENTS**

I would like to thank my mentor, Dr. Edward Yeh for his advice, guidance and support. His methods of mentoring allow me to develop as an independent scientist and critical thinker. I would also like to thank all my supervisory committee members, Dr. Chen Dong, Dr. Shao-Cong Sun, Dr. Phillip Carpenter and Dr. Sue-Hwa Lin for their inspiration, guidance and support. I am very grateful to Dr. Pornpimon (Yee) Angkasekwinai for her instructions and collaborative efforts relating to basic immunology techniques such as FACS and cell sorting, and for her scientific discussion during research collaboration.

I would like to thank my former advisor Dr. Chengming Zhu for his mentoring in DNA repair and tumorigenesis in mouse models. I am very thankful to my former advisory committee members, Dr. Phillip Carpenter, Dr. Elsa R. Flores, Dr. Randy J. Legerski and Dr. Honnavara N. Ananthaswamy for their advice, discussion and encouragement.

I would like to thank the Yeh lab members, Dr. Jinke Cheng, Dr. Sui Zhang, Dr. Hong Dou, Dr. Tasneem Bawa, Dr. Lu Long-Sheng, Dr. Qi Yitao, Dr. Jingxiong Wang, Dr. Chao Huang and Robert Nguyen for their scientific discussion, friendship and encouragement. I would also like to thank my friends at GSBS, particularly Omar Duramad, Bon Trinh, Hoai Nam Nguyen-Jackson and Catherine T. Nguyen for their encouragement. I would also thank the flow cytometry core facility (Karen Martinez, David He, and Amy Cortez) of MD Anderson Cancer Center for technical assistance. Finally, I would like to acknowledge Vietnam Education Foundation for giving me the opportunity to pursue my Ph.D. in the US.

**SUMO-SPECIFIC PROTEASE 1 CONTROLS  
LYMPHOID DEVELOPMENT THROUGH REGULATION OF  
SUMOYLATION/ACETYLATION SWITCH IN STAT5**

Publication No. \_\_\_\_\_

Thang Van Nguyen, D.V.M., M.S.

Supervisory Professor: Edward T.H. Yeh, M.D.

SUMOylation has emerged as an important regulatory mechanism for protein function. SUMO-specific proteases (SENPs) are essential for removing SUMO from conjugated proteins in many different systems, but the physiological functions of SENPs are poorly understood. STAT5 (Signal Transducer and Activator of Transcription 5) plays a critical role in the development of lymphoid cells. However, it is not known whether STAT5 is regulated by the SUMOylation pathway. Here, we showed that SUMOylated STAT5 is accumulated in SENP1<sup>-/-</sup> lymphoid precursors. SENP1 deficiency results in severe defects in early T and B cell development, similar to that observed in mice harboring a complete inactivation of STAT5. Because STAT5 is SUMOylated and acetylated at the same lysine residue, SENP1 deficiency blocks STAT5 in the SUMOylation state, resulting in diminished STAT5 acetylation and phosphorylation, and defective lymphoid development. Thus, our results reveal a novel function of SENP1 in the regulation of early lymphoid development via an acetylation/SUMOylation switch in STAT5.

## TABLE OF CONTENTS

APPROVAL.....	i
TITLE PAGE.....	ii
DEDICATION.....	iii
ACKNOWLEDGEMENTS.....	iv
ABSTRACT.....	v
TABLE OF CONTENTS.....	vi
LIST OF FIGURES.....	viii
<b>INTRODUCTION.....</b>	<b>1</b>
SUMO	1
The SUMOylation pathway	2
Biological Function of SUMO-specific proteases	5
Molecular mechanisms by which SUMOylation regulates transcription	8
STAT proteins	10
Regulation of STATs by acetylation	11
Regulation of STATs by PIASs	13
Regulation of STATs by SUMOylation	14
Biological function of STATs	15
STAT1	15
STAT2	16
STAT3	16
STAT4	17

STAT6	18
STAT5	18
Lymphoid development	19
<b>AIM OF THE PRESENT STUDY.....</b>	<b>25</b>
<b>RESULTS.....</b>	<b>26</b>
SENP1 is highly expressed at the early stages of T and B lymphocytes	26
SENP1 deficiency impairs T cell development	26
SENP1 deficiency impairs B cell development	33
SENP1 deficiency does not affect myeloid cell development	36
SENP1 deficiency leads to intrinsic defects in T and B development	40
SENP1 regulates SUMOylation of STAT5	43
SUMOylation inhibits tyrosine phosphorylation of STAT5	51
SENP1 controls an acetylation/SUMOylation switch in STAT5	58
<b>DISCUSSION.....</b>	<b>65</b>
SENP1 is essential for early lymphoid development	65
STAT5 is regulated by the SUMOylation pathway	67
SENP1 regulate STAT5 SUMOylation	68
The acetylation/SUMOylation switch in STAT5 is regulated by SENP1	70
Proposed model	71
<b>MATERIALS AND METHODS.....</b>	<b>74</b>
<b>REFERENCES.....</b>	<b>81</b>
<b>VITA.....</b>	<b>101</b>



## LIST OF FIGURES

Figure 1: SUMOylation vs deSUMOylation	4
Figure 2: The lymphoid developmental pathway	21
Figure 3: The T cell development pathway	22
Figure 4: The B cell development pathway	23
Figure 5: The IL-7R signaling pathway	24
Figure 6: SENP1 mRNA expression in hematopoietic lineage populations	27
Figure 7: SENP1 deficiency affects the thymic development	28
Figure 8: SENP1 deficiency results in a severe defect in early T cell development	30
Figure 9: Myeloid development in OP9-DL1 coculture	31
Figure 10: T cell development in OP9-DL1 coculture after 14 days	32
Figure 11: SENP1 deficiency affects B cell development during embryonic development	34
Figure 12: SENP1 deficiency results in a severe defect in early B cell development	35
Figure 13: Myeloid development in OP9 coculture after 7 days	37
Figure 14: B cell development in OP9-DL1 coculture after 14 days	38
Figure 15: SENP1 deficiency does not affect myeloid development	39
Figure 16: SENP1 deficiency impairs T cell development in fetal liver transplantation experiments	41

Figure 17: SENP1 deficiency impairs B cell development in fetal liver transplantation experiments	42
Figure 18: The expression of key transcription factors involved in lymphoid differentiation	45
Figure 19: The expression of IL-7R components	45
Figure 20: Modified STAT5 accumulated in SENP1 <sup>-/-</sup> B and T cells	46
Figure 21: Endogenous STAT5 is SUMOylated by endogenous SUMO2/3 <i>in vivo</i>	47
Figure 22: Both STAT5A and STAT5B are SUMOylated in an over-expression system	48
Figure 23: SUMOylation of STAT5 is enhanced by PIAS3	49
Figure 24: SENP1 de-SUMOylates SUMOylated STAT5 <i>in vivo</i>	50
Figure 25: Schematic diagram of full-length murine STAT5 protein structure	53
Figure 26: STAT5 is not SUMOylated at lysine residues within the SUMO consensus motifs	54
Figure 27: mapping STAT5 SUMOylation sites	55
Figure 28: Tyrosine phosphorylation of STAT5 is diminished in SENP1 <sup>-/-</sup> B and T cells	56
Figure 29: SUMOylation of STAT5 is tyrosine phosphorylation-dependent	57
Figure 30: The K696R mutant of STAT5 impairs its transcriptional activity	60
Figure 31: STAT5 is acetylated by p300	61
Figure 32: Lysine 696 is also a target for acetylation	62
Figure 33: Endogenous STAT5 is acetylated in FL cells treated with IL-7	63
Figure 34: SENP1 regulates the SUMOylation/acetylation status of STAT5	64

Figure 35: A model for the role of SENP1 in the regulation of  
acetylation-SUMOylation switch in STAT5

72

## INTRODUCTION

### **SUMO (small ubiquitin-like modifier)**

In eukaryotes, protein function is tightly regulated by multiple mechanisms, such as control of transcription and translation, and post-translational modifications. Over the past decade, posttranslational modifications of proteins, including phosphorylation, methylation, acetylation and ubiquitination, have been extensively studied. SUMOylation, a posttranslational modification of proteins by SUMO, has emerged recently as an important regulatory mechanism in cell physiology.

SUMO was discovered in the mid 1990s. The *Saccharomyces cerevisiae* *SMT3* gene was first described as a suppressor of MIF2 protein required for mitotic spindle integrity in 1995 (1). In 1996, using the death domain in the cytoplasmic tail of Fas/APO-1 and TNF receptor 1 as bait in the yeast two-hybrid system, our laboratory reported the isolation of a novel death domain-interacting protein, Sentrin (later re-named as SUMO-1), which exhibited homology to ubiquitin, Nedd8 and SMT3. Interestingly, Overexpression of Sentrin protected cells from both anti-Fas/APO-1 and TNF-induced cell death. However, the mechanism by which Sentrin inhibits the cell death signaling remains to be elucidated (2). At the same time, five other groups identified different human and mouse homologs of *S. cerevisiae* *SMT3*, and called them different names, including PIC1 (PML interacting protein 1), GMP1 (GAP-modifying protein 1), Ubl1 (ubiquitin-like protein 1), SUMO-1 (small-ubiquitin-related modifier) (3-6), and human HSMT3 (later called human SUMO-2)

(7). Although human SUMO-1 has only a low sequence similarity of 18% to ubiquitin, NMR structure analysis revealed that SUMO-1 and Ubiquitin display similar 3-D structure (8). The C-terminal diglycine residues of both SUMO and ubiquitin are required for formation of an isopeptide bond with the  $\epsilon$ -NH<sub>2</sub> group of lysine residue in targeted proteins.

Four SUMO homologs have been identified in mammalian cells. SUMO-1 shares 47% similarity at the protein level with SUMO-2 and 3, whereas SUMO-2 and 3 are 95% identical. In contrast to SUMO-1, SUMO-2 and SUMO-3 have a consensus SUMOylation site at their N-terminal region and can form poly-SUMO Chains (9). SUMO-1, 2 and 3 are ubiquitously expressed in all tissues, while SUMO-4 expression is limited to immune tissues and kidney (10, 11). The conjugation of SUMO-4 to substrates *in vivo* still remains obscure.

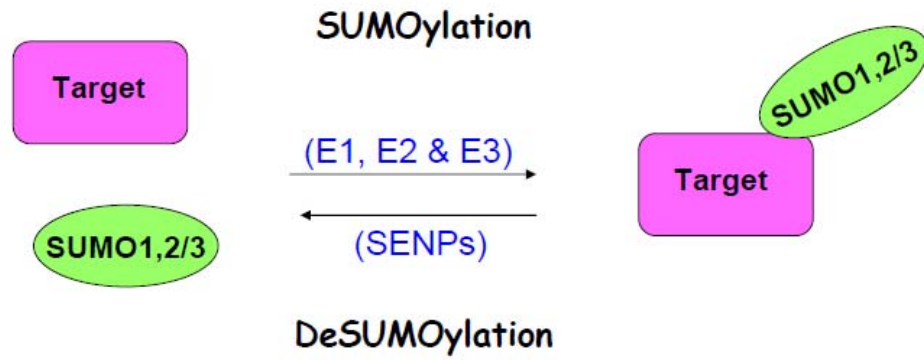
### **The SUMOylation pathway**

All SUMO proteins are synthesized as immature forms that must be processed to expose the C-terminal di-glycine motif to generate the mature forms. This processing is carried out by the sentrin/SUMO-specific protease (SENP) family. The mature form of SUMOs is activated by a SUMO-activating enzyme (E1), a heterodimer containing SAE1 (SUMO-activating enzyme subunit 1) and SAE2, transferred by a SUMO-conjugating enzyme (E2) UBC9, and in some cases, to a SUMO protein ligase (E3) that catalyzes the formation of an isopeptide bond between the C-terminal carboxy group of SUMO and the  $\epsilon$ -amino group of lysine in

protein substrates. Lysine residues subject to SUMO modification are usually found within a SUMO modification consensus motif,  $\psi$ KXE/D (where  $\psi$  is a large hydrophobic residue and X is any residue). Currently, several SUMO E3 ligases have been characterized, including PIAS (protein inhibitor of activated STAT) proteins, the nucleoporin RanBP2 and the polycomb protein Pc2 (12, 13).

The covalent modification of proteins by SUMO can be reversed by SENPs, as illustrated in Figure 1. Thus, SENP regulates SUMOylation via (1) its C-terminal hydrolase activity required for removing C-terminal amino acids from SUMO to reveal the diglycine residues important for conjugation to SUMO substrates and/or (2) its isopeptidase activity that is essential for removing SUMO from conjugated substrates. Two yeast SENPs, including Ulp1 and Ulp2/Smt4 were characterized first (14, 15). Ulp1 has both the C-terminal hydrolase activity and the isopeptidase activity, while Ulp2 possesses only the isopeptidase activity.

In 2000, our laboratory cloned the first mammalian Sentrin/SUMO-specific protease (SEN1), which is distantly related to the yeast Ulp1 (16). Currently, six SENPs have been identified in mammalian cells. They can be divided into three subfamilies on the basis of their sequence homology, cellular localization and substrate specificity (17-19). The first subfamily, consisting of SEN1 and SEN2, can process either SUMO-1 or SUMO-2/3-conjugated proteins. The second subfamily contains SEN3 and SEN5, which prefer SUMO2/3 as substrates and are localized in the nucleolus. The third subfamily including SEN6 and SEN7 also favors SUMO2/3-modified substrates.



**Figure 1. SUMOylation vs deSUMOylation.** Target protein can be SUMOylated by SUMO E1, E2 and E3 ligases, and SUMO-conjugated protein can be deSUMOylated by Sentrin/SUMO-specific proteases (SENPs) to remove SUMO from target.

## Biological Function of SUMO-specific proteases

SUMOylation has been shown to play key roles in regulating diverse cellular processes, including transcription, DNA repair, cell-cycle progression and signal transduction from yeast to human (13, 19). SENPs are essential for reversing SUMOylation in many different systems; however, the physiological significance and *in vivo* functions of SENPs are poorly understood.

In an attempt to understand the role of SENP1 in development, Yamaguchi et al. generated mice from an ES cell line with a retroviral vector that was randomly inserted into the enhancer region on the *SENP1* gene (20). This random insertion reduced expression of the *SENP1* transcript, resulting in embryonic lethality between E12.5 and E14.5. Interestingly, the mutation caused the accumulation of the SUMOylated forms of a number of proteins both in the fetus and placenta. These data suggest that SENP1 is important for regulating the levels of SUMOylated proteins during embryonic development. However, the exact molecular mechanism by which SENP1 contributes to development is not clear.

Because SENP1 expression in Yamaguchi's mouse model is partially blocked, our laboratory generated complete SENP1 knockout mice to further evaluate the contribution of SENP1 in development. SENP1 knockout (KO) mice were generated from an embryonic stem cell line with a gene trap vector that was inserted into the mouse SENP1 open reading frame at codon 310 (21). This insertion completely blocked SENP1 expression. SENP1 deficiency caused embryonic lethality between E13 and E15, due to severe fetal anemia (21). SENP1 controls EPO



production by regulating the stability of hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ). In the absence of SENP1, hypoxia induced HIF1 $\alpha$  SUMOylation, thereby leading to HIF1 $\alpha$  ubiquitylation and proteasomal degradation.

Recently, another group created SENP1 KO mice based on a Cre-loxP system (22). SENP1<sup>lox/lox</sup> mice were crossed with  $\beta$ -actin-Cre mice to mediate a recombination *in vivo*, resulting in a complete deletion of SENP1 exon 5 and exon 6 and a frameshift of the downstream catalytic domain. This global deletion of SENP1 leads to embryonic lethality between embryonic day 13.5 and postnatal day 1, and anemia (22). SENP1 controls GATA1 activation and erythropoiesis through deSUMOylation of GATA1.

Two groups have independently uncovered the role of SENP2 and the importance of SUMOylation in development. Chiu and colleagues generated SENP2 KO mice using the Cre-LoxP system and demonstrated that SENP2<sup>-/-</sup> mice exhibited embryonic lethality between E10.5 to E11.5, due to a placenta defect (23). In the absence of SENP2, SUMOylated Mdm2 accumulates in the nucleus, where it is unable to promote p53 degradation. Accumulation of p53 in trophoblast progenitors causes cell growth arrest and subsequent defect in placental development. Our laboratory generated SENP2 KO mice using a gene trap vector inserted into intron 10 of *SENP2* genomic DNA and disrupted the transcript of the catalytic domain of SENP2 (24). Deletion of the murine *SENP2* gene causes embryonic lethality at E10, due to a profound defect in the cardiac development (24). The expression of Gata4 and Gata6 genes is essential for cardiac development, and is regulated by occupancy of Pc2/CBX4, a polycomb repressive complex 1 (PRC1) subunit, on their promoters.

In the absence of SENP2, SUMOylated Pc2/CBX4 accumulates and Pc2/CBX4 occupancy at the promoters of PcG target genes is markedly increased, leading to repression of *Gata4* and *Gata6* transcription (24). The phenotypic differences observed in these two SENP2 KO mouse models are difficult to explain. It is possible that the KO strategies used to inactivate the *SENP2* gene may also contribute to the difference in phenotypes observed. Future studies using tissue specific gene-targeting (Cre/loxP system) to inactivate the *SENP2* gene are needed to further characterize the role of SENP2 and its target genes in development.

A more recent study by Dou et al. has revealed that SENP6 regulates the SUMOylation status of RPA70, which plays a critical role in DNA repair through homologous recombination (HR) (25). SENP6-depleted cells exhibited the typical phenotypes of DNA damage response, including the accumulation of  $\gamma$ -H2AX foci, activated cell cycle check points (phosphorylated Chk1 and Chk2) and defective cell cycle progression in both S phase and G<sub>2</sub>/M phases, correlating with the accumulation of SUMOylated RPA70. Biochemical experiments further indicated that RPA70 SUMOylation serves as HR mediator to facilitate recruitment of Rad51 to the DNA damage foci to initiate DNA repair through HR. This study clearly demonstrates the role of SENP6 in regulating RPA70 SUMOylation and HR. However, suppression of SENP6 accumulated DNA damage foci, suggesting that SENP6 is also essential for maintaining genomic stability, possibly through regulation of DNA replication or telomere elongation/maintenance. Further investigations are required to clarify this important issue.

## **Molecular mechanisms by which SUMOylation regulates transcription**

SUMOylation plays a critical role in regulating gene expression by various mechanisms. Instead of single-site action, many proteins are dynamically modified at multiple sites by different modifications, including phosphorylation, acetylation, ubiquitination, and sumoylation. This phenomenon is known as multisite modification (26). Hietakangas and others identified a PDSM (phosphorylation-dependent sumoylation motif), containing a SUMO consensus site and an adjacent proline-directed phosphorylation site ( $\Psi KxExxSP$ ) (27, 28). This highly conserved motif regulates phosphorylation-dependent sumoylation of multiple substrates, such as heat-shock factors (HSFs), GATA-1, and myocyte enhancer factor 2 (MEF2). Thus, SUMOylation can affect other modifications or function of proteins.

Since a lysine residue can be a target of different posttranslational modifications, SUMOylation can block alternative lysine-targeted modifications such as ubiquitination, methylation or acetylation. Ubiquitination and proteasome-mediated degradation of I $\kappa$ B $\alpha$  are known to be crucial for activation of NF- $\kappa$ B. In 1998, Hay's group demonstrated that I $\kappa$ B $\alpha$  is SUMOylated on K21, which is also targeted by ubiquitination (29). SUMOylated I $\kappa$ B $\alpha$  cannot be ubiquitinated and is therefore resistant to proteasome-mediated degradation. Thus, by blocking ubiquitination, SUMOylation creates a privileged pool of I $\kappa$ B $\alpha$  that is resistant to signal-induced degradation (29). Transcriptional activity of transcription factors, such as SP3 and MEF2A, can be regulated by interplay between SUMOylation and

acetylation on the same lysine residue (30, 31). Interestingly, an acetylation-sumoylation switch on MEF2 is further regulated by its phosphorylation (31).

Many transcription factors, coactivators, or corepressors are SUMOylated, resulting in alterations of their cellular localization, stability, and interaction with binding partners. In most cases, SUMOylated proteins suppress transcription, possibly due to their interaction with co-repressors. Therefore, blocking SUMO conjugation to substrates by lysine-to-arginine mutation of SUMOylation sites or by overexpression of SENPs can increase the transcriptional activity of transcription factors (32).

An increasing number of studies support the notion that SUMOylation of proteins alters their interaction with binding partners, in particular those having a SUMO-interaction motif (SIM) (33, 34). The SIM mediates non-covalent interactions between SIM-containing proteins and SUMO. This motif consists of the short hydrophobic peptide sequence V/I/L-V/I/L-X-V/I/L or V/I/L-X-V/I/L-V/I/L and in some cases, are preceded, or followed, by a stretch of acidic amino acids. A recent study by Glass group has highlighted the importance of SIM in regulation of Toll-like receptors (TLRs) in macrophages (35). Transcriptional activation of many TLR-responsive genes is archived by an initial de-repression step that nuclear receptor co-repressor (NCoR) complexes are actively removed from the promoters of target genes. Ligand-dependent SUMOylation of liver X receptors (LXRs) inhibits TLR4-induced transcription potently by preventing the NCoR clearance step. The study has demonstrated that SUMOylated LXRs block NCoR turnover by binding to

a conserved SUMO2/SUMO3-interaction motif in CORO2A, a component of the NCoR complex, and preventing nuclear actin recruitment.

Another example of SIM is SUMO-targeted ubiquitin Ligase (STUbL) family of proteins, which selectively ubiquitinates sumoylated proteins and proteins containing SUMO-like domains (SLDs). STUbL recruitment to sumoylated/SLD proteins is mediated by tandem SUMO interaction motifs (SIMs) within the STUbLs N-terminus (36, 37). Although the roles of STUbLs in regulating transcription and DNA repair in yeast have been documented, little is known about their functions in mammalian cells.

### **STAT proteins**

It is well known that cytokines play a central role in the regulation of the immune system. Three key families of transcription factors that are widely used downstream of cytokine-mediated to control gene expression include the signal transducer and activator of transcription (STAT) family of proteins, nuclear factor- $\kappa$ B (NF- $\kappa$ B) and SMA (small body size)-and MAD (mothers against decapentaplegic)-related proteins (SMADs) (38). In 1992, STAT1 and STAT2 were the first members of STAT proteins identified as mediators of the cellular response to interferons (IFNs) (39, 40). Currently, seven mammalian STATs have been identified, including STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6, encoded by different genes (41). In the human genome, *Stat1* and *Stat4* genes are clustered together on chromosome 2, and *Stat3*, *Stat5a* and *Stat5b* genes

are clustered together on chromosome 17, whereas *Stat2* and *Stat6* genes are clustered together on chromosome 12 (42). All STAT proteins share six structural regions: an N-terminal domain (ND), a coil-coil domain (CC), a DNA-binding domain (DBD), a linker domain (L), a Src homology 2 (SH2) domain, and a transcriptional activation domain (TAD) at the carboxy (C) terminus. A single tyrosine residue (Y700) located in the TAD is required for STAT activation.

The JAK (Janus kinase)-STAT signaling pathway consists of three main components: a receptor, JAK and STAT. Tyrosine kinases of the JAK family, including tyrosine kinase 2 (TYK2), JAK1, JAK2 and JAK3, are physically associated with the intracellular domains of many cytokine receptors. After cytokines bind to their cognate receptor on the cell surface, JAKs are activated and they then phosphorylate the cytoplasmic tails of the cytokine receptors at tyrosine residues, leading to recruitment and phosphorylation of STATs. Subsequently, activated STATs in the cytoplasm translocate to the nucleus, where they bind to specific DNA sites and activate the transcription of their specific genes.

### **Regulation of STATs by acetylation**

In eukaryotic cells, chromatin is composed of DNA, histones and other nuclear proteins, and can be broadly classified into condensed heterochromatin and decondensed euchromatin. DNA in heterochromatin is inaccessible to enzymes for processes such as transcription, recombination and DNA repair while euchromatic DNA is accessible and active for these processes. Constitutive heterochromatin is

formed at repetitive DNA sequences, such as pericentromeres and telomeres, whereas the formation of facultative heterochromatin is inducible, and is often associated with a change in cell phenotype.

A variety of modifications at the N-terminal tail of histones, such as acetylation, methylation and phosphorylation form the basis of the “histone code”, which can act as either repressive or active marks (43). These histone modifications recruit distinct protein complexes that affect chromatin structure. For histone lysine methylation (mono-, di-, and tri-methylation), H3 methylated at lysine-4 (K4), K36, and K79 are enriched in active chromatin, whereas methylation of H3-K9, H3-K27, and H4-K20 is responsible for heterochromatic silencing (44-46). Acetylation of histones H3 and H4 is associated with transcriptionally active chromatin. The role of histone lysine modifications in transcription regulation is well established; however modifications of non-histone proteins, in particular by acetylation, have recently emerged as an important mechanism of epigenetic regulation in transcription (47).

The histone acetyltransferases CREB-binding protein (CBP)/p300 have been shown to interact with various STAT family members within both the C-terminal TAD and N-terminal domain, and promote STAT-mediated transcription (48-51). This raised the possibility that STATs could be modified by acetylation. Several studies have been reported concerning the acetylation of different STAT family members. STAT6 was the first STAT protein shown to be a target of acetylation (52). STAT1 is modified by acetylation at lysines 410 and 413. Interestingly, a phosphorylation-acetylation switch regulates STAT1 signaling (53, 54). Recent studies by Chin and colleagues reveal that STAT3 is acetylated at lysine 685 by

p300/CBP and that this modification is essential for STAT3 to form stable dimers and to activate transcription (55), whereas STAT2 acetylation on Lys390 regulates STAT2:STAT1 interaction (56), and STAT5 acetylation is required for dimerization (57). These studies clearly indicate that modification of STATs by acetylation is critical for regulating the JAK-STAT signaling pathway.

### **Regulation of STATs by PIAS (Protein Inhibitor of Activated STAT)**

The mammalian protein inhibitor of activated STAT (PIAS) family of proteins was initially identified as negative regulators of STAT signaling (58, 59). The PIAS family includes PIAS1, PIAS3, PIASx (also known as PIAS2) and PIASy (also known as PIAS4). Except for PIAS1, each PIAS protein has two isoforms. Biochemical studies have revealed that more than 60 proteins, most of them transcription factors, can be regulated by members of the PIAS family through multiple mechanisms (38). First, a PIAS protein might block the DNA-binding activity of a transcription factor. A study by Shuai's group showed that *Pias1* Deletion enhanced the antiviral activity of IFN- $\alpha$ - or IFN- $\beta$  (60). As a result, *Pias1*<sup>-/-</sup> mice showed increased protection against pathogenic infection. This is probably because PIAS1 selectively regulates a subset of IFN- $\alpha$ - or IFN- $\beta$ -inducible genes by interfering with the recruitment of STAT1 to the gene promoter (60). Secondly, a PIAS protein might recruit other co-regulators, such as histone deacetylases (HDACs), to repress transcription. For example, Shuai's group has recently reported



that PIAS1 binding to the Foxp3 promoter leads to recruitment of DNA methyltransferases and heterochromatin protein 1 for epigenetic modifications (61). Pias1 deletion resulted in promoter demethylation, reduced histone H3 methylation at Lys9, and enhanced promoter accessibility. As a result, Pias1<sup>-/-</sup> mice showed an increased natural CD4(+)Foxp3(+) regulatory T (T(reg)) cell population and were resistant to the development of experimental autoimmune encephalomyelitis (61). Thirdly, a PIAS protein might repress transcription by promoting the sumoylation of a transcription factor. Lastly, PIAS proteins might repress transcription by altering the localization of transcription factors. Despite their inhibitory roles in transcriptional regulation, PIAS proteins can regulate positively several transcription factors. For example, PIAS3 activates, whereas PIASy represses, the transcriptional activity of SMAD3 (62).

### **Regulation of STATs by SUMOylation**

PIAS proteins have been shown to function as SUMO-specific E3 ligases (63). This raises the possibility that STATs might be regulated by the SUMOylation pathway. Several groups have reported that STAT1 is SUMOylated on Lys703 in an overexpression system and that its SUMOylation can be greatly enhanced by PIAS proteins (63, 64). However, the *in vivo* function of SUMOylation in the regulation of STAT1 activity remains unknown. In addition, it is not known whether other STAT proteins can be modified by SUMO.

## **Biological function of STATs**

To elucidate the *in vivo* function of STAT proteins, all STAT genes have been gene-targeted in mice. The phenotypes of these mice reveal distinctive functions of STAT genes and the importance of these genes in regulating the development and function of the immune system (65-68).

### **STAT1**

IFNs are known to regulate cellular antiviral, antiproliferative and immunological responses. IFNs include two types of IFNs, Type-I (IFN- $\alpha$  and IFN- $\beta$ ) and Type-II (IFN- $\gamma$ ), which bind to the Type-I IFN receptor and the Type-II IFN receptor, respectively. IFN- $\alpha/\beta$  activates both STAT1 and STAT2, whereas IFN- $\gamma$  stimulates the tyrosine phosphorylation of STAT1 but not STAT2. *STAT1*<sup>-/-</sup> mice are viable and fertile, but highly susceptible to microbial and viral infections and tumor formation due to severely impaired IFN responses (69, 70). In addition to IFNs, Stat1 can be activated by many growth factors including IL-6, IL-10, growth hormone and thrombopoietin. However, no major developmental defects were observed in *STAT1*<sup>-/-</sup> mice involving non-IFN signaling. Thus, STAT1 is essential for the IFN-dependent signaling pathways.

## **STAT2**

As discussed above, the major function of STAT2 is involved in IFN- $\alpha/\beta$  signaling. *STAT2* knockout mice are fertile and viable but exhibit a number of defects in immune response, including an increased susceptibility to viral infection and the loss of IFN- $\alpha/\beta$  responsiveness (71).

## **STAT3**

STAT3 can be activated by IL-6 family members, IFN $\gamma$ , IL-10, and granulocyte-colony stimulating factor (G-CSF). Targeted deletion of STAT3 causes embryonic lethality at E7.5, suggesting that STAT3 plays a role during early embryogenesis (72). Increasing evidence suggests that STAT3 plays critical roles in the immune system, including inhibition of macrophage inflammatory signaling, control of dendritic cell differentiation, regulation of steady state and emergency granulopoiesis and regulation of B cell development (73-76). For example, Akira's group generated STAT3 conditional KO mice by breeding of mice carrying a *loxP*-flanked Stat3 allele with LysMcre mice in which the Cre-recombinase is expressed under control of the murine lysozyme M gene regulatory region (73). In these mice, STAT3 is deficient specifically in macrophages and neutrophils. The mutant mice exhibit increased production of inflammatory cytokines such as TNF- $\alpha$ , IL-1, and IFN- $\gamma$  and impaired suppressive effects of IL-10 on inflammatory cytokine production from macrophages and neutrophils (73).

It was initially thought that after activation by professional antigen-presenting cells (APCs), naïve CD4<sup>+</sup> T cells were able to differentiate into effector cells, which could be divided into two subsets, T helper 1 (Th1) and T helper 2 (Th2) cells based on their cytokine-expression profiles and immune-regulatory function (77). Recently, three other subsets have been identified. The first new subset of T<sub>H</sub> cells is CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells (T reg cells), which play a central role in the maintenance of immune tolerance (78-80). Dong and colleagues have characterized two other novel T<sub>H</sub>-cell subsets, including follicular helper T cells and IL-17-producing CD4<sup>+</sup> T (T<sub>H</sub>17) cells (also identified by Weaver's group), which have crucial roles in immune regulation (81, 82). Interestingly, STAT3 is crucial for the differentiation of the T<sub>H</sub>17 cells (83). In contrast, activation of STAT5 is a critical factor in the induction and maintenance of FOXP3 expression in Treg cells (84).

## **STAT4**

IL-12 promotes naïve CD4<sup>+</sup> T cells (Th0 cells) to differentiate into Th1 cells, which secrete IL-2 and IFN- $\gamma$ , and play a critical role in cellular-mediated immunity. STAT4 is activated by IL-12. *STAT4*<sup>-/-</sup> mice are viable and fertile. However, these mice showed impaired Th1 differentiation, IFN- $\gamma$  production and cell-mediated immunity (85, 86), similar to the phenotypes of mice lacking IL-12 or IL-12R subunits.

## **STAT6**

STAT6 is activated in response to by IL-4 and IL-13. In addition, STAT6 is essential for differentiation of Th2 cells important for humoral immunity. Accordingly, *STAT6*<sup>-/-</sup> mice showed defects in IL-4-mediated functions including Th2 helper T-cell differentiation, expression of cell surface markers, and immunoglobulin class switching to IgE (87, 88).

## **STAT5**

STAT5 was first isolated as a prolactin-induced transcription factor named mammary gland factor (MGF) in mammary gland tissue (89). STAT5 consists of two highly related genes encoding STAT5A and STAT5B proteins, which are 96% similar at the amino acid level (90). Activated by a number of cytokines, growth factors, and hormones, including IL-2, IL-3, IL-5, IL-7, IL-9, IL-15, GM-CSF, thrombopoietin, erythropoietin, and growth hormone, STAT5 has been implicated in various cellular processes, such as hematopoiesis, immunoregulation, reproduction, and lipid metabolism. Among all the STAT family members, only STAT5 has been demonstrated to play an essential role in regulating both T and B cell development (90).

## **Lymphoid development**

All hematopoietic cells are derived from hematopoietic stem cells (HSCs). According to a classical model, HSCs differentiate into common lymphoid progenitors (CLPs), which give rise to both B and T cells (91), as shown in Figure 2.

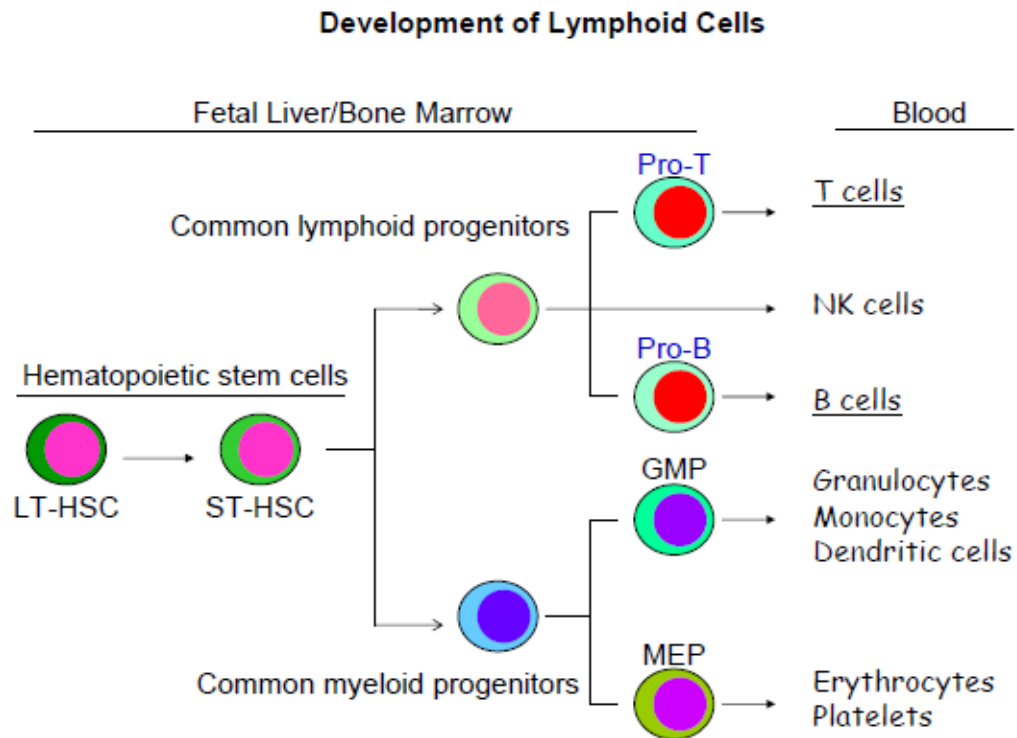
Recent studies have suggested that HSCs differentiate into either common myeloid progenitors (CMPs) or lymphoid-primed multipotent progenitors (LMPPs) in the bone marrow (BM) (92). Myeloid cells stem from either CMPs or LMPPs, while erythroid cells and megakaryocytes develop from CMPs (93). Lymphocytes arise from LMPPs, which differentiate into the earliest lymphocyte progenitors (ELPs) (94). Subsequently, ELPs differentiate into common lymphoid progenitors (CLPs) and early thymic progenitors (ETPs) from which B and T cell development occurs in the BM and thymus, respectively. During embryonic development, hematopoietic stem cells arise in the aorta/gonad/mesonephros (AGM) region and migrate to the fetal liver where hematopoiesis takes places.

As illustrated in Figure 3, thymocyte development proceeds through multiple stages, which can be characterized by the sequential expression of cell surface markers. The early immature T cells are CD4<sup>-</sup>CD8<sup>-</sup> double-negative (DN) thymocytes. They can be divided into four different developmental stages, defined based on their surface expression of CD44 and CD25 (95). Similarly, B cell development also occurs through various stages, including CLPs, pre-proB, early pro-B, late pro-B, pre-B, immature B and mature B cells. Each stage can be characterized by their expression of cell-surface markers, such as B220, CD43,

CD19, BP-1, HAS, IgM and IgD, and can be defined according to the Hardy nomenclature (Figure 4) (96).

The development of T and B cells is tightly regulated by multiple transcription factors and cytokines (97, 98). IL-7 (Interleukin-7) is a non-redundant and essential cytokine for the development of both T and B cells (99). Its receptor consists of two chains, IL-7R $\alpha$  and common cytokine receptor  $\gamma$ -chain ( $\gamma$ C), which binds the Janus kinase 3 (Jak3) (100, 101). The  $\gamma$ C chain is expressed in various hematopoietic lineages, whereas the IL-7R $\alpha$  subunit is preferentially expressed in the lymphoid system from CLPs to large pre-B and DN1-DN3 T cells as well as in thymic and peripheral CD4<sup>+</sup> and CD8<sup>+</sup> single-positive (SP) T cells (102).

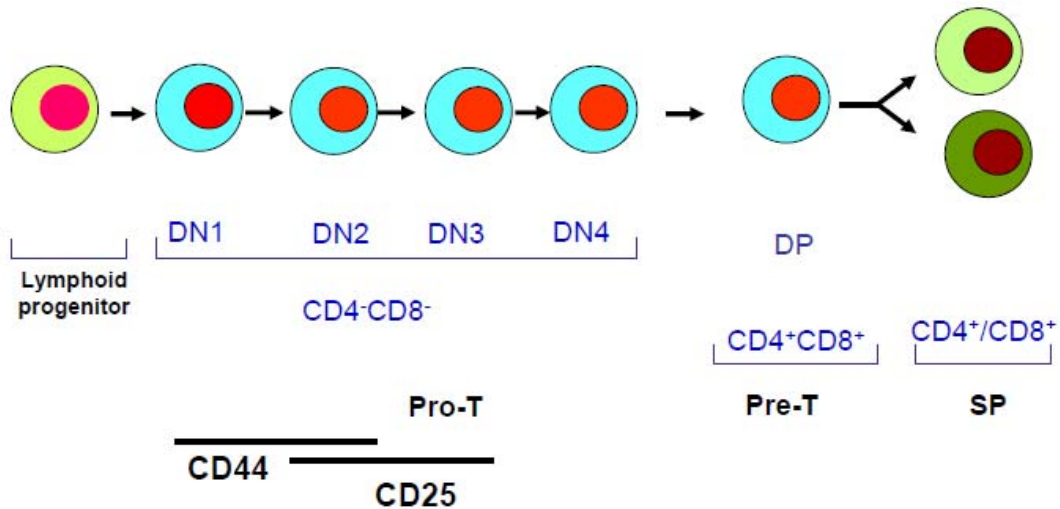
Binding of IL-7 to its receptor triggers activation of Jak1 and Jak3, resulting in STAT5 activation, subsequent translocation to the nucleus and regulation of target gene transcription (Figure 5) (103). STAT5 contains two highly related isoforms, STAT5A and STAT5B, which are encoded by separate genes and play a critical role in the development and function of immune cells (90).



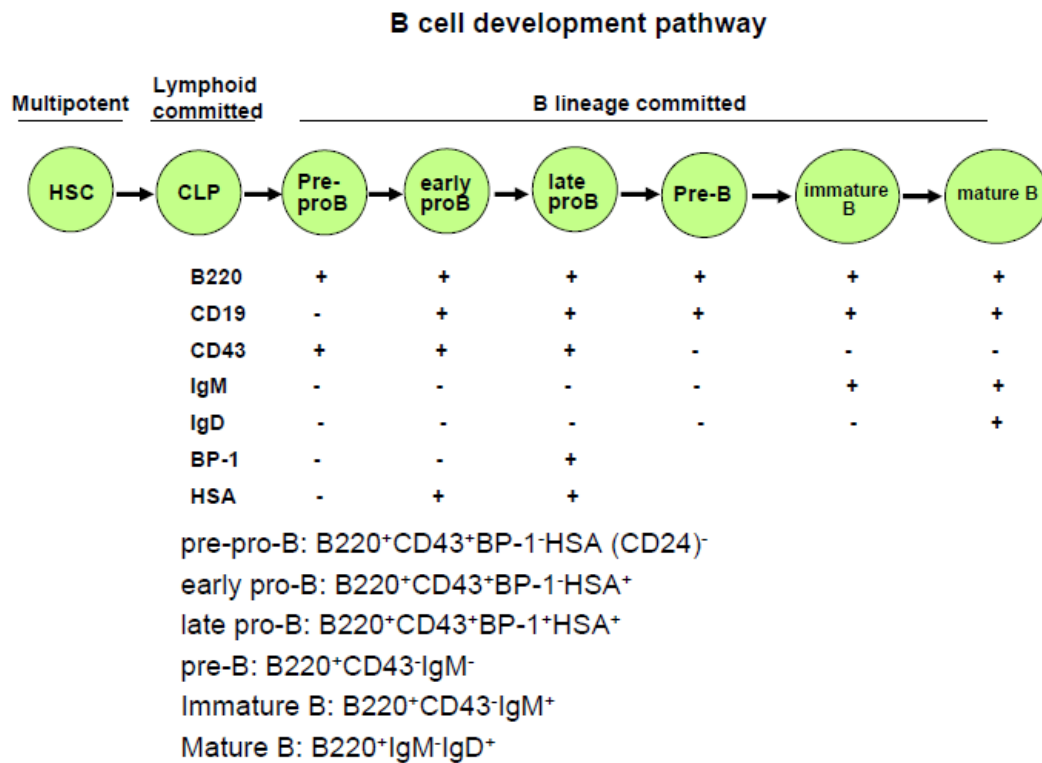
**Figure 2. The lymphoid developmental pathway.** Both B and T cells are differentiated from hematopoietic stem cells through multiple stages including common lymphoid progenitors, pro- and pre-B/T cells, before becoming mature B/T cells.



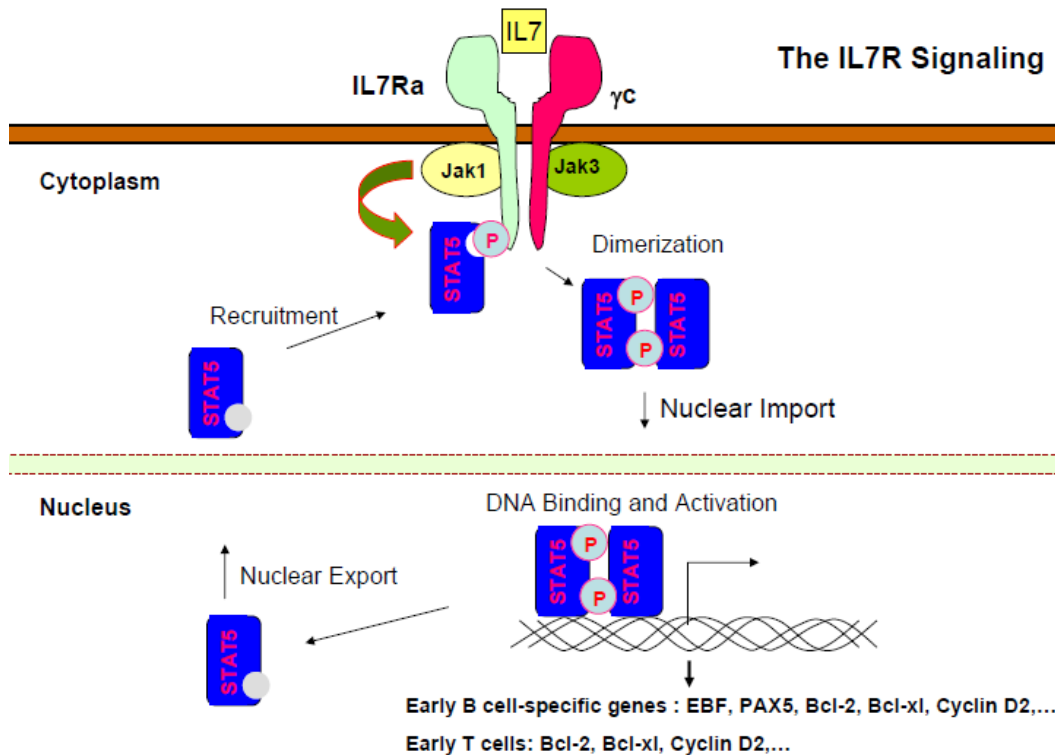
### T cell development in the thymus



**Figure 3. The T cell development pathway.** T cell development proceeds through multiple stages, which can be characterized by the sequential expression of cell surface markers, including CD44, CD25, CD4 and CD8. The early immature T cells are CD4<sup>-</sup>CD8<sup>-</sup> double-negative (DN) thymocytes, which can be divided into four different developmental stages (DN1-4).



**Figure 4. The B cell development pathway.** B cell development occurs through various stages, which can be characterized by their expression of cell-surface markers according to the Hardy nomenclature (Hardy and Hayakawa, 2001).



**Figure 5. The IL-7R signaling pathway.** In unstimulated state, STAT5 resides in the cytoplasm. Binding of ligand (IL7) to its receptor leads to phosphorylation of the cytoplasmic tail of receptor, resulting in recruitment of STAT5 to the receptor. STAT5 is phosphorylated by JAK kinases. Phosphorylated STAT5 dimerizes and translocates to the nucleus, where it turns on the transcription of STAT5-specific B/T cells and then returns back to the cytoplasm.

## AIM OF THE PRESENT STUDY

SUMOylation has been shown to regulate a number of cellular processes, including transcription, DNA repair, cell-cycle progression and signal transduction from yeast to human (18, 19). Many transcription factors are SUMOylated, resulting in alteration of their function (32). Previously, we reported that inactivation of the murine *SENP1* gene results in embryonic lethality prior to embryonic day 16.5 (E16.5), due to a severe defect in definitive erythropoiesis stemming from deficient Epo production (21). SENP1 KO mouse study revealed that SENP1 controls EPO production by regulating the stability of HIF1 $\alpha$  (21). Despite extensive biochemical studies on SENPs *in vitro*, the physiological significance and *in vivo* functions of the SENPs remain poorly understood. In particular, the physiological function of SENP1 in the development of lymphoid cells has not been defined. The overall aim of my PhD dissertation was to investigate the role of SENP1 in T and B lymphopoiesis using SENP1 KO mice that we described earlier (21).

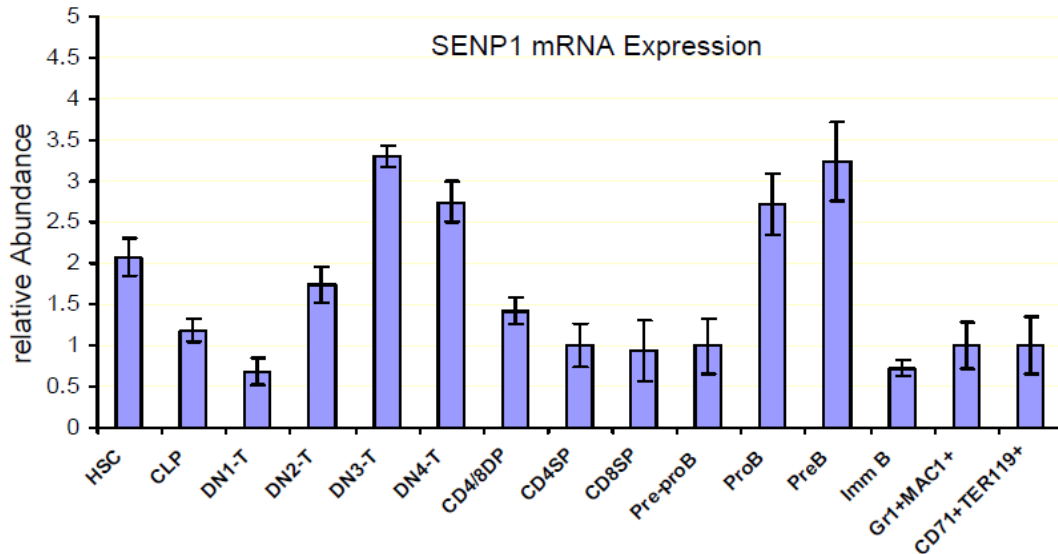
## RESULTS

### **SENP1 is highly expressed at the early stages of T and B lymphocytes**

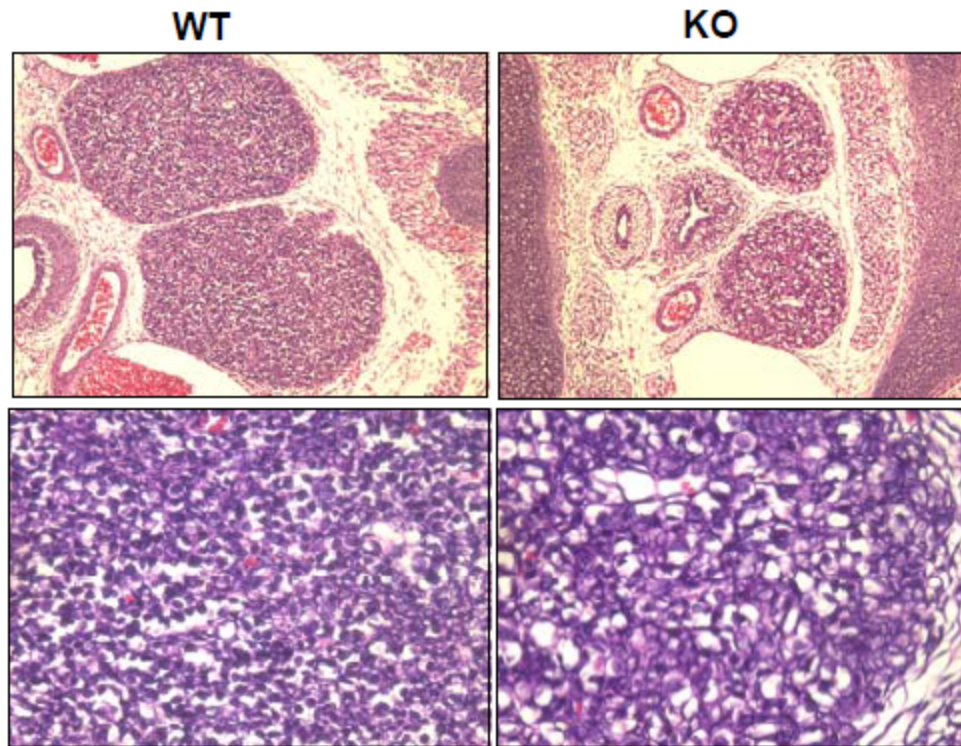
To investigate the role of SENP1 in lymphopoiesis, we first analyzed its expression pattern in lymphoid cells. Interestingly, SENP1 was highly expressed at the early stages of T and B cell development including DN2, DN3 and DN4 T cells and, pro-B and Pre-B cells, respectively (Figure 6), raising the possibility that SENP1 might participate in T and B lymphopoiesis.

### **SENP1 deficiency impairs T cell development**

We next examined the phenotype of thymus in SENP1<sup>-/-</sup> embryos. Histological examination of thymic sections revealed that both the size and cellularity of E15.5 SENP1<sup>-/-</sup> thymi were markedly decreased compared with those of wild-type littermates (Figure 7). To check whether a partial or complete block in T cell development could explain thymic hypocellularity and smaller thymus size in SENP1<sup>-/-</sup> embryos, we cocultured hematopoietic stem cells (HSCs) isolated from fetal livers (FL) in E14.5 wild-type and SENP1<sup>-/-</sup> embryos with OP9 stromal cells expressing Delta-like 1 (OP9-DL1 cells) in the presence of cytokines Flt3L (FMS-like tyrosine kinase 3 ligand) and IL-7 (Interleukin-7) to induce T cell differentiation *in vitro* (104).



**Figure 6. SENP1 mRNA expression in hematopoietic lineage populations.** Each population was isolated by flow-sorting from the thymus and bone marrow of 4- to 6-week-old C57BL/6 mice. SENP1 mRNA expression was measured by quantitative RT-PCR using TaqMan assay kit from ABI. Gene expression was normalized to 18s rRNA level. The relative abundance is displayed as an average of triplicates of quantitative PCR in each sample, and error bars indicate  $\pm$  SD.

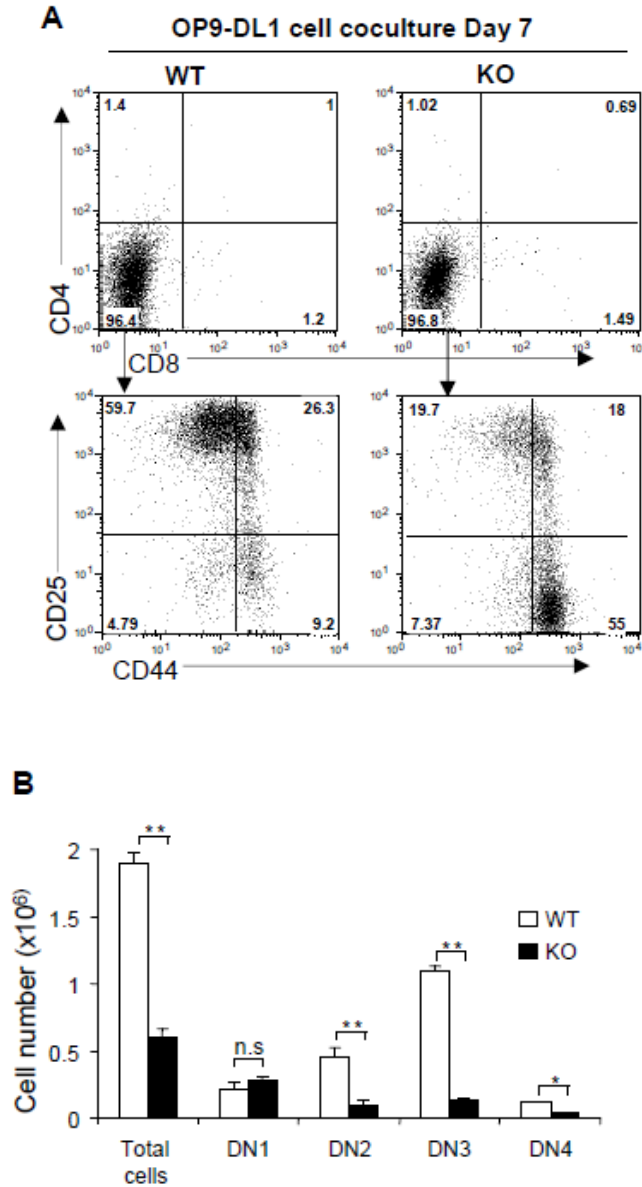


**Figure 7. SENP1 deficiency affects the thymic development.** Hematoxylin and eosin staining of thymic sections (top panel, original magnification: 100x and bottom panel, original magnification: 400x) from wild-type (WT) and  $SENP1^{-/-}$  (KO) embryos at E15.5. The results shown are a representative of at least four independent experiments.

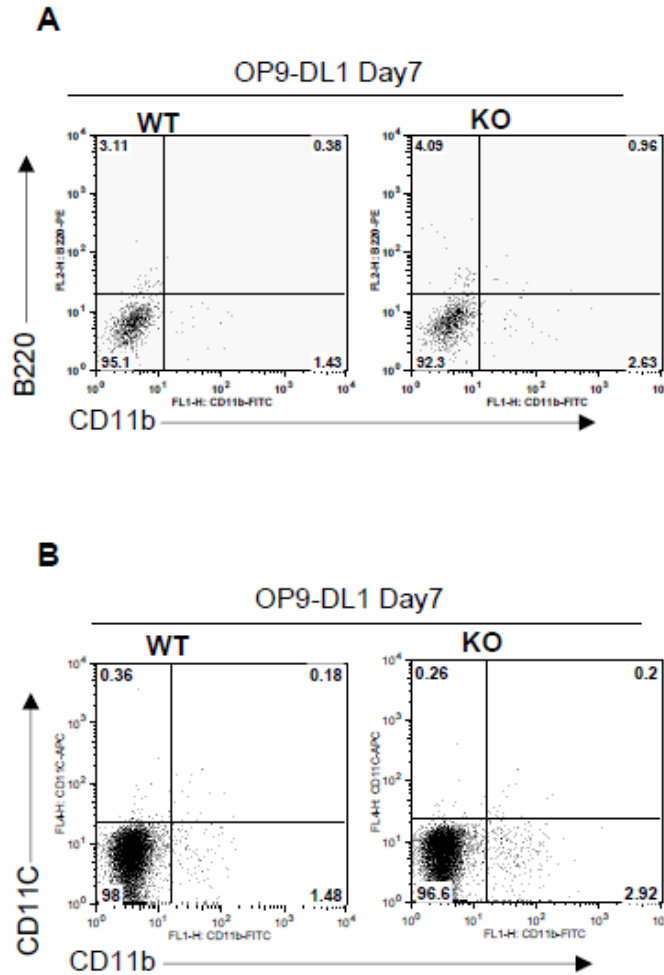
After 7 days of co-culture, flow cytometric analysis revealed that most of the cells derived from both wild-type and SENP1<sup>-/-</sup> HSCs were immature CD4<sup>+</sup>CD8<sup>+</sup> (DN) thymocytes (Figure 8A, top panel). However, further segregation of DN thymocytes into distinct subsets (DN1-DN4) showed an increased frequency of DN1 subset, and decreased frequencies of DN2 and DN3 subsets generated from SENP1<sup>-/-</sup> HSCs (Figure 8A, lower panel). Furthermore, the absolute numbers of DN2, DN3 and DN4 T cells derived from SENP1<sup>-/-</sup> HSCs were significantly decreased compared with those of wild-type controls (Figure 8B). These results indicate that SENP1 is required for early T cell development at or before the DN2 stage.

Under OP9-DL1 cell co-culture conditions, wild-type and SENP1<sup>-/-</sup> HSCs were unable to generate B and myeloid cells (Figures 9A and 9B). After 14 days of coculture on OP9-DL1 cells, despite a substantial decrease in the absolute cell numbers, CD4/CD8 double positive (DP), CD4 single positive (SP) and CD8 SP could still be generated from SENP1<sup>-/-</sup> HSCs (Figure 10). These results strongly suggest that SENP1 deficiency leads to an incomplete block in early T cell development.

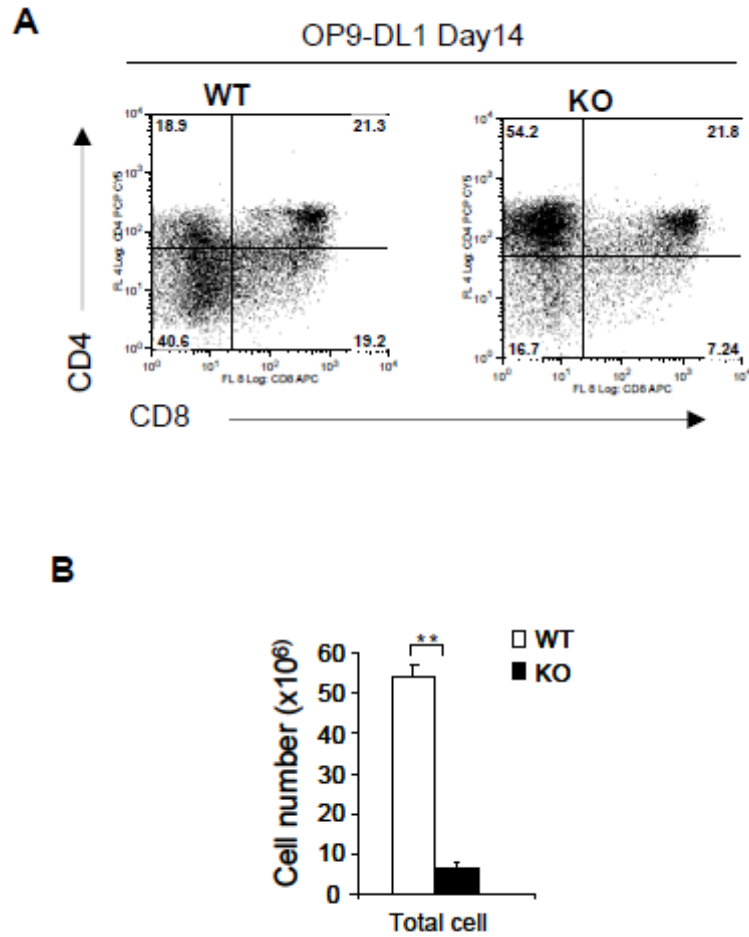




**Figure 8. SENP1 deficiency results in a severe defect in early T cell development.** (A) FL-HSCs ( $\text{Lin}^- \text{c-Kit}^+ \text{Sca1}^+$ ) from wild-type (WT) and  $\text{SENP1}^{-/-}$  (KO) embryos at E14.5 were cocultured on OP9-DL1 stromal cells in the presence of IL-7 and Flt3L. After 7 days of coculture, cells were isolated and analyzed by flow cytometry for T cell markers CD4 and CD8 (top panel). Gated  $\text{CD4}^+ \text{CD8}^+$  population was further analyzed based on CD44 and CD25 (lower panel). Data shown are representative of three independent experiments. (B) Absolute numbers of the indicated subpopulations of thymocytes were calculated based on flow cytometry (shown in B) and total thymocytes counts, and represented as mean  $\pm$  SD;  $n=5$  each. \*,  $P<0.05$ ; \*\*,  $P<0.01$ ; n.s. is not significant.



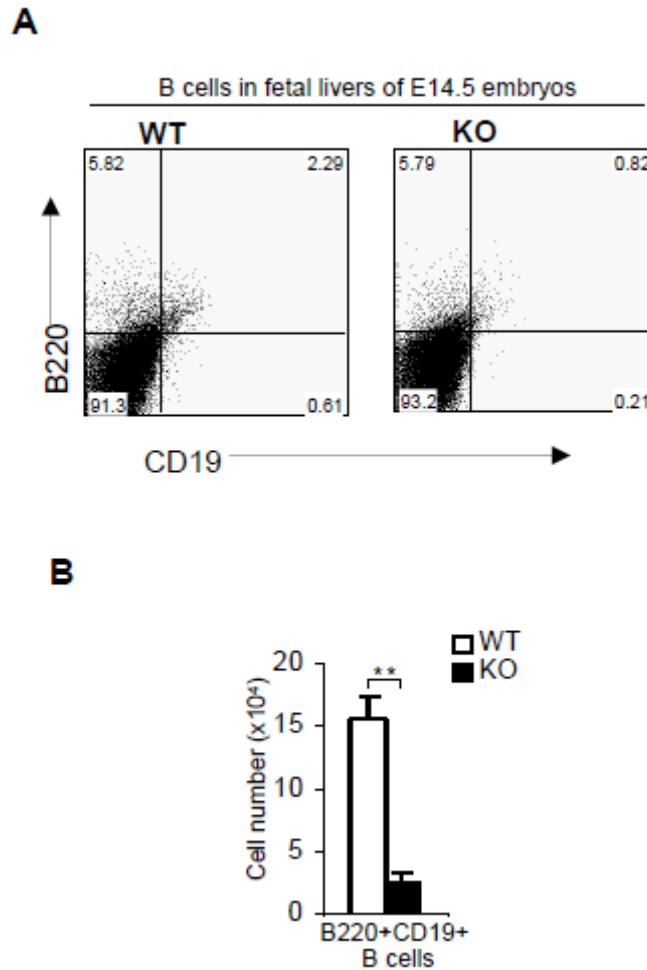
**Figure 9. Myeloid development in OP9-DL1 coculture. (A-B)** Both wild-type and  $SENPI^{-/-}$  HSCs cocultured on OP9-DL1 cells were unable to generate B and myeloid cells. Sorted FL-HSCs were cultured on OP9-DL1 stromal cells in the presence of IL-7 and Flt3L for 7 days. Flow cytometric analysis was performed using B220 and CD11b (A) antibodies, and CD11C and CD11b antibodies (B). Data shown are representative of three independent experiments.



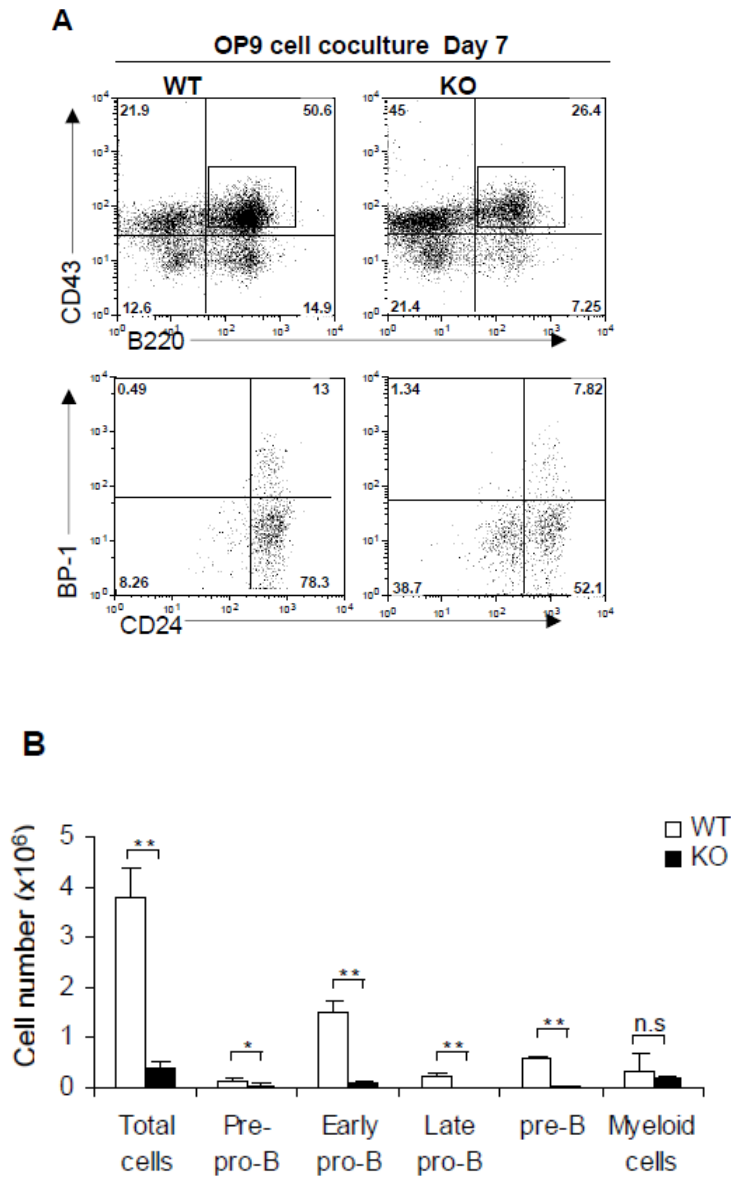
**Figure 10. T cell development in OP9-DL1 coculture after 14 days.** (A-B) Wild-type and *SENP1*<sup>-/-</sup> FL-HSCs were cultured on OP9-DL1 stromal cells in the presence of IL-7 and Flt3L for 14 days. T cells were analyzed by flow cytometry using CD4 and CD8 antibodies (A). Absolute cell number was counted and presented as means  $\pm$  SD; n=5 each (B). \*\*, P<0.01.

## SENP1 deficiency impairs B cell development

Next, we investigated whether B cell development is also affected by the absence of SENP1. We first examined B lymphopoiesis in fetal livers from E14.5 SENP1<sup>-/-</sup> embryos and found that SENP1<sup>-/-</sup> embryos displayed a severe reduction in the absolute number of B220<sup>+</sup>CD19<sup>+</sup> B cells compared with wild-type littermate controls (Figures 11A and 11B). To further assess the effects of SENP1 deficiency on B cell development, we co-cultured FL-HSCs with OP9 stromal cells in the presence of Flt3L and IL-7 to promote B cell differentiation *in vitro* (105). After 7 days of coculture, flow cytometric analysis revealed an increased frequency of early pro-B (B220<sup>+</sup>CD43<sup>+</sup>BP-1<sup>-</sup>CD24<sup>+</sup>) cells generated from wild-type HSCs. In contrast, most B cells generated from SENP1<sup>-/-</sup> HSCs displayed markers of pre-pro-B (B220<sup>+</sup>CD43<sup>+</sup>BP-1<sup>-</sup>CD24<sup>-</sup>) and early pro-B (B220<sup>+</sup>CD43<sup>+</sup>BP-1<sup>-</sup>CD24<sup>+</sup>) cells (Figure 12A). Furthermore, the absolute numbers of pre-pro-B, early pro-B, late pro-B and pre-B cells derived from SENP1<sup>-/-</sup> HSCs were significantly reduced compared with wild-type controls (Figure 12B), suggesting a critical role of SENP1 at the earliest stages of B cell development.



**Figure 11. SENP1 deficiency affects B cell development during embryonic development.** (A-B) Fetal liver cells were harvested from E14.5 wild-type and SENP1<sup>-/-</sup> embryos. B cells were analyzed by staining with B220 and CD19 antibodies (A), absolute cell numbers of B220+CD19+ B cells were calculated and presented as means  $\pm$  SD (B); n=5 each. \*\*, P<0.01.

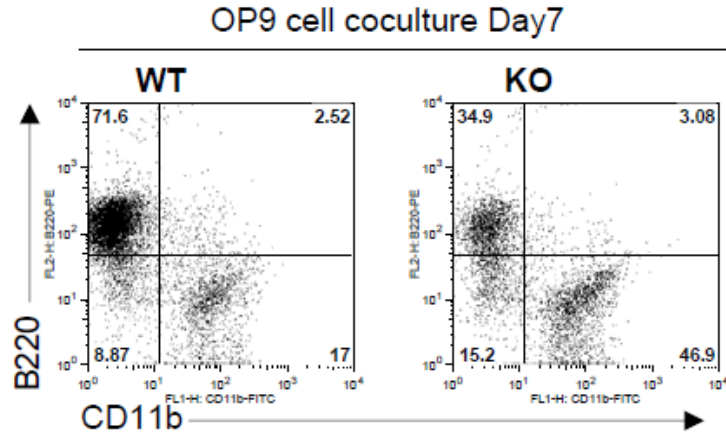


**Figure 12. SENP1 deficiency results in a severe defect in early B cell development.** (A) Sorted FL-HSCs from wild-type and  $SENP1^{-/-}$  embryos at E14.5 were cocultured on OP9 stromal cells in the presence of IL-7 and Flt3L. After 7 days of coculture, cells were isolated and analyzed by flow cytometry for B cell markers B220 and CD43 (top panel), CD24 and BP-1 (Gated  $CD43^{+}B220^{+}$  population, lower panel). Data shown are representative of three independent experiments. (B) Absolute number of B cells in each developmental stage was calculated based on flow cytometry (shown in D) and total cell number, and represented as mean  $\pm$  SD;  $n=5$  each. \*,  $P<0.05$ ; \*\*,  $P<0.01$ ; n.s. is not significant.

Under OP9 cell coculture conditions, myeloid development was not affected in the absence of SENP1 since the absolute number of myeloid cells derived from both wild-type and SENP1<sup>-/-</sup> HSCs remained similar (Figures 12B and 13). After 14 days of coculture, we also found significant reductions in the frequencies and absolute numbers of pro-B and pre-B cells (B220<sup>+</sup>CD19<sup>+</sup>) (Figures 14A and 14B), and immature B cells (B200<sup>+</sup>IgM<sup>+</sup>) derived from SENP1<sup>-/-</sup> (Figures 14C and 14D). These results clearly showed that SENP1 is required for B cell development at the early stages.

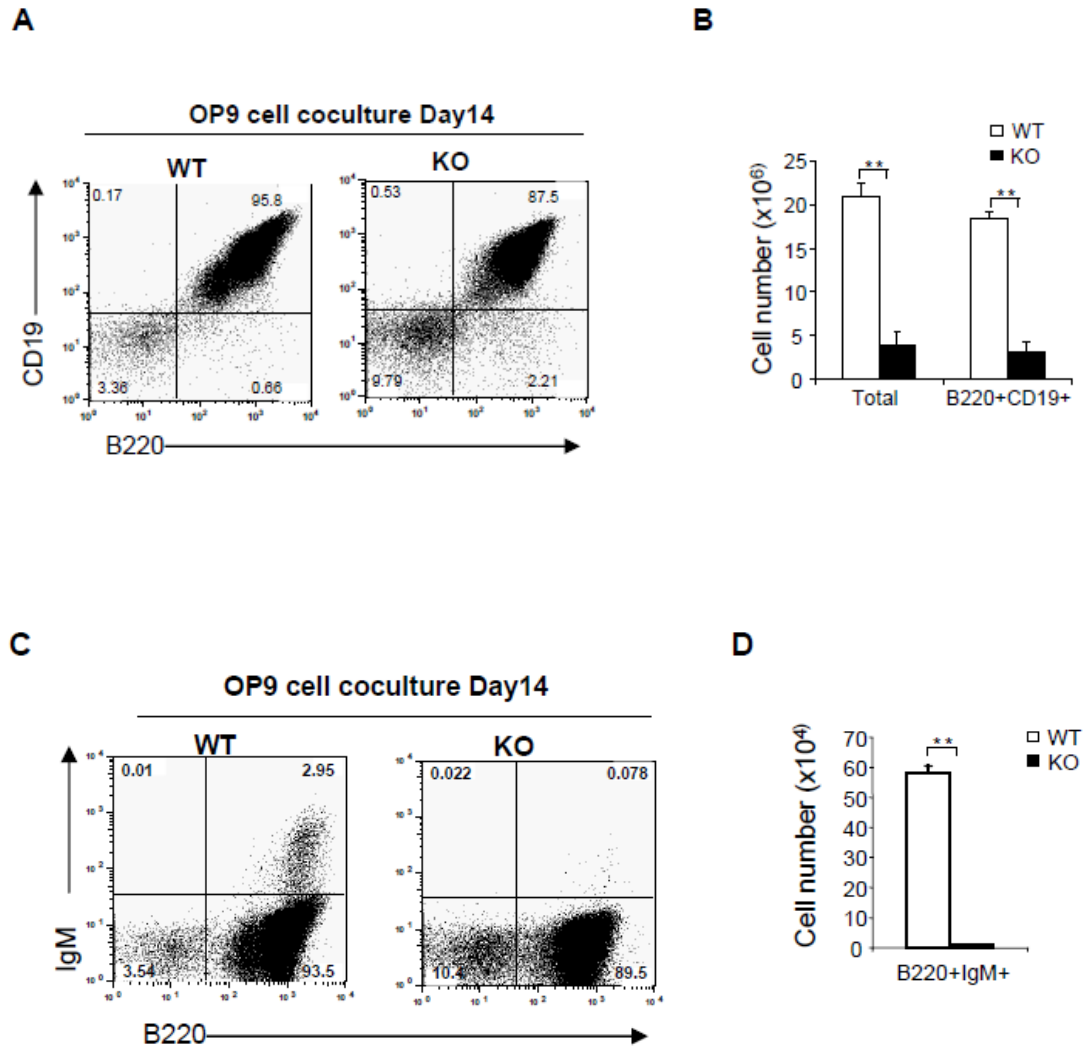
### **SENP1 deficiency does not affect myeloid cell development**

To demonstrate that the hematopoietic defects caused by SENP1 deficiency are restricted to the erythroid (21) and lymphoid lineages, but not the myeloid lineage, we co-cultured wild-type and SENP1<sup>-/-</sup> HSCs with OP9 stromal cells in the presence of IL3, IL6, SCF, and Flt3L, and analyzed myeloid cells expressing Gr-1 and CD11b markers after 7 days of culture. Consistent with our previous report (21), no defect in myeloid-lineage development was observed in the absence of SENP1 since the frequency and absolute number of myeloid cells (Gr-1<sup>+</sup>CD11b<sup>+</sup>) generated from both wild-type and SENP1<sup>-/-</sup> HSCs were comparable (Figures 15A and 15B).

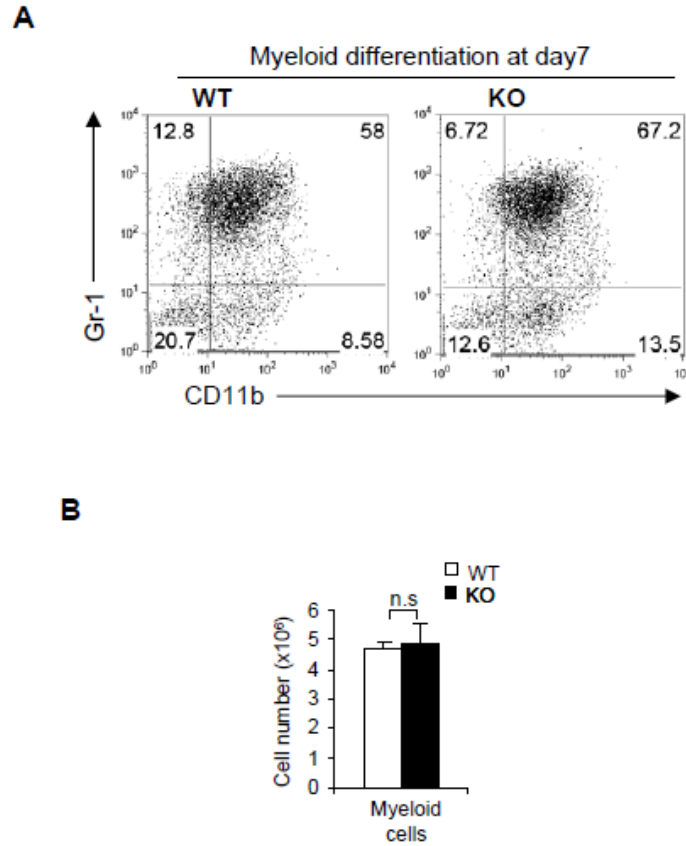


**Figure 13. Myeloid development in OP9 coculture after 7 days.** Sorted FL-HSCs from wild-type and  $SENPI^{-/-}$  embryos at E14.5 were cocultured on OP9 stromal cells in the presence of IL-7 and Flt3L. After 7 days of coculture, cells were isolated and analyzed by flow cytometry for B cell marker (B220), and myeloid cell marker CD11b. Absolute number of myeloid cells was calculated and shown in Figure 7B. Data shown are representative of three independent experiments.





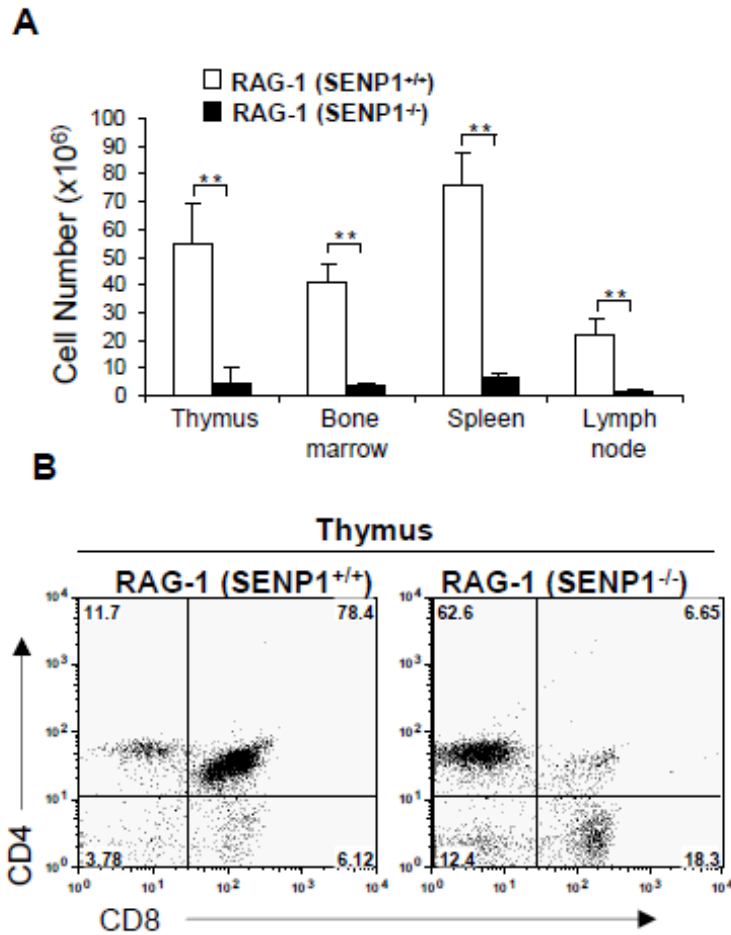
**Figure 14. B cell development in OP9-DL1 coculture after 14 days.** (A-D) Wild-type and *SEN1*<sup>-/-</sup> FL-HSCs were cocultured on OP9 stromal cells in the presence of IL-7 and Flt3L for 14 days. B cells were analyzed by flow cytometry using B220 and CD19 antibodies (A), and B220 and IgM antibodies (B), and their absolute cell numbers were calculated and presented as means  $\pm$  SD (C and D, respectively); n=5 each. \*\*, P<0.01. Data shown are representative of three independent experiments.



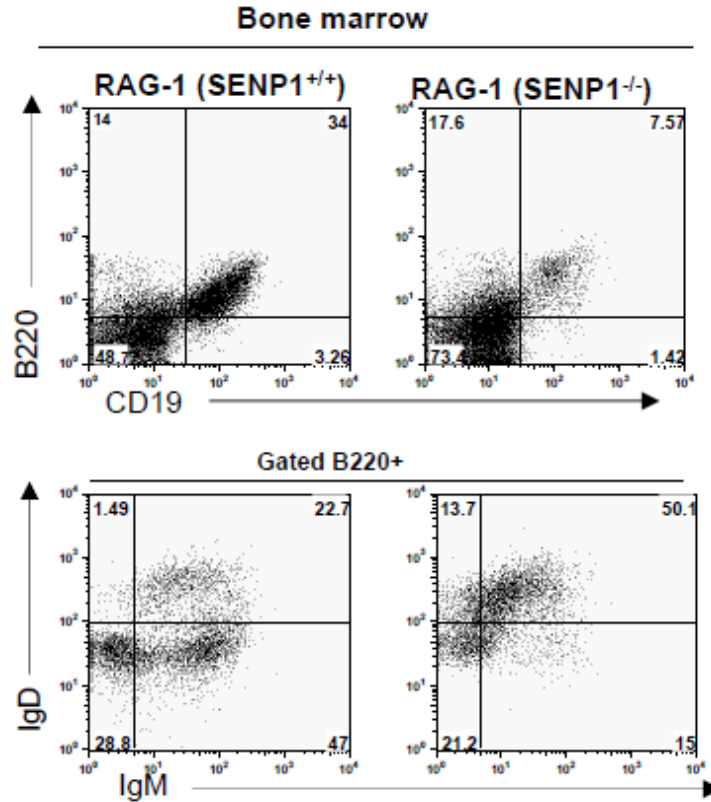
**Figure 15. SENP1 deficiency does not affect myeloid development.** (A-B) Wild-type and SENP1<sup>-/-</sup> FL-HSCs were cultured on OP9 stromal cells in the presence of IL3 (10ng/ml), IL6 (10ng/ml), SCF (50 ng/ml), and Flt3L (50 ng/ml) for 7 days. Myeloid cells were analyzed by flow cytometry using indicated antibodies (A). Data shown are representative of three independent experiments. The absolute cell numbers of myeloid cells (Gr-1<sup>+</sup>CD11b<sup>+</sup>) were calculated based on flow cytometry and total cell counts, and presented as means  $\pm$  SD; n=5 each. n.s. is not significant (B).

### **SENP1 deficiency leads to intrinsic defects in T and B development**

To further confirm that SENP1 deficiency resulted in defects that are intrinsic to T and B cell development, we performed FL transplantation experiments using RAG-1<sup>-/-</sup> mice, which lack mature B and T cells (106). FL cells from E14.5 wild-type and SENP1<sup>-/-</sup> embryos were transplanted into lethally irradiated RAG-1<sup>-/-</sup> mice. Six weeks after transplantation, the lymphoid compartments of the recipients were harvested, counted and analyzed by flow cytometry. Compared with RAG-1<sup>-/-</sup> mice reconstituted with wild-type FL cells (wild-type recipients), the total cell numbers in lymphoid organs of RAG-1<sup>-/-</sup> mice reconstituted with SENP1<sup>-/-</sup> FL cells (SENP1<sup>-/-</sup> recipients), exhibited a 10- to 20-fold reduction (Figure 16A). Analysis of T cell development in the thymi of recipients showed that the frequency of DP T cells in the thymi of SENP1<sup>-/-</sup> recipients was greatly reduced compared with wild-type recipients (Figure 16B). Similarly, analysis of B cell development in the bone marrow of recipients revealed that the frequencies of CD19<sup>+</sup>B220<sup>+</sup> and B220<sup>+</sup>IgM<sup>+</sup>IgD<sup>-</sup> B cells were significantly reduced in SENP1<sup>-/-</sup> recipients (Figure 17). Taken together, our data demonstrated that SENP1 deficiency results in intrinsic defects in T and B development.



**Figure 16. SENP1 deficiency impairs T cell development in fetal liver transplantation experiments.** (A) Cell counts in lymphoid compartments including thymus, bone marrow, spleen and lymph node of RAG1<sup>-/-</sup> mice reconstituted with wild-type and SENP1<sup>-/-</sup> fetal liver cells. Total cell number was calculated and represented as mean  $\pm$  SD; n= 6 each. \*\*, P<0.01. (B) Flow cytometric analysis for T cell markers including CD4 and CD8 in the thymi of RAG1<sup>-/-</sup> mice reconstituted with wild-type and SENP1<sup>-/-</sup> fetal liver cells. Data shown are representative of two independent experiments.



**Figure 17. SENP1 deficiency impairs B cell development in fetal liver transplantation experiments.** Flow cytometric analysis of B cells in bone marrow from RAG1<sup>-/-</sup> mice reconstituted with wild-type and SENP1<sup>-/-</sup> fetal liver cells using B cell markers B220 and CD19 (upper panel). Gated B220<sup>+</sup> cells were further analyzed based on IgM and IgD (lower panel). Data shown are representative of two independent experiments.

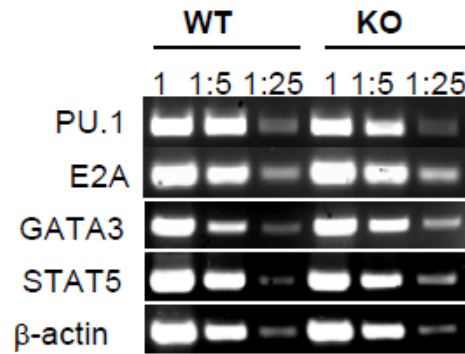
## SENP1 regulates SUMOylation of STAT5

To check whether SENP1 deficiency may affect transcription of key transcription factors crucial for early lymphoid development, we analyzed their expression in fetal livers of E14.5 embryos by RT-PCR. Expression levels of PU.1, STAT5, GATA3 and E2A from SENP1<sup>-/-</sup> fetal livers remained unchanged compared with those from wild-type littermate controls (Figure 18). To determine whether the impaired development of B and T cells in SENP1 deficiency is due to reduced expression of IL-7R components, real-time PCR analysis was performed. The mRNA levels of IL-7R components including IL-7R $\alpha$  and  $\gamma_c$  chains, Jak1 and Jak3 in fetal livers of E14.4 SENP1<sup>-/-</sup> embryos were undisturbed, suggesting that the expression of genes involved in the IL-7R signaling pathway is intact (Figure 19). Indeed, the lymphoid defects in SENP1 deficiency described here are quite similar to the one observed in STAT5 deficiency (107-109).

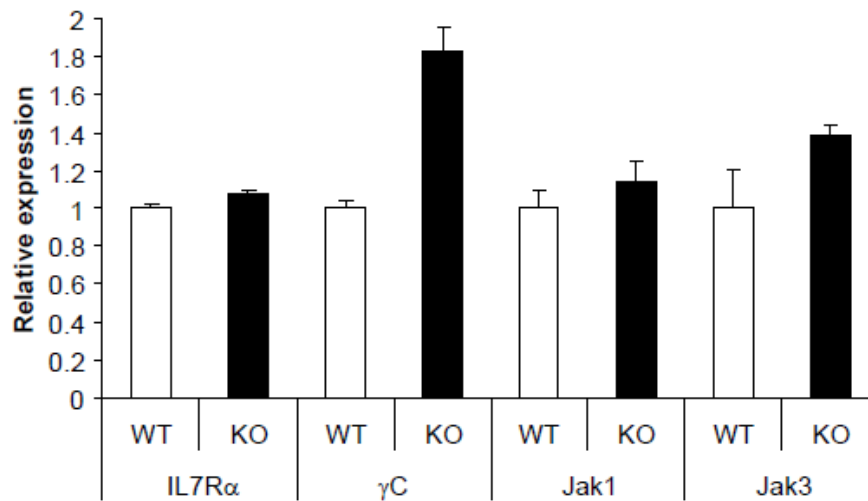
Given that SENP1 regulated activity of transcription factors (21, 22), we therefore hypothesized that SENP1 may control early development of T and B cells through altering the SUMOylation status of STAT5. To explore this possibility, we first examined SUMOylation of STAT5 in wild-type and SENP1<sup>-/-</sup> B, T and myeloid cells derived from HSC differentiation *in vitro* after 7 days of coculture by Western blot analysis. As shown in Figure 20, the ~95-kDa full-length STAT5 was detected in all wild-type and SENP1<sup>-/-</sup> B, T and myeloid cells, whereas a modified form of STAT5 migrating at 135 kDa (~ 40 kDa shift) was observed only in SENP1<sup>-/-</sup> B and T cells. On the other hand, the protein level of another STAT family member,

STAT3 in SENP1<sup>-/-</sup> B and T cells was unchanged compared with wild-type B and T cells, respectively. To verify that the ~ 135-kDa band is a SUMOylated form of STAT5, lysates of wild-type and SENP1<sup>-/-</sup> B cells were subjected to denaturing immunoprecipitation with anti-STAT5 antibody, followed by immunoblotting with anti-SUMO-1 and anti-SUMO-2/3 antibodies. This ~ 135-kDa band displayed in SENP1<sup>-/-</sup> B cells was readily detected with anti-SUMO-2/3, but not by anti-SUMO-1 or control IgG (Figure 21). These results clearly demonstrate that the modified form of STAT5 accumulated in SENP1<sup>-/-</sup> B and T cells is indeed SUMOylated STAT5. Consistent with the accumulation of SUMOylated STAT5, the expression of BCL-2, a downstream target of STAT5 was markedly decreased in SENP1<sup>-/-</sup> B and T cells compared with wild-type B and T cells, respectively (Figure 20, third panel). These data suggest that deletion of SENP1 results in the accumulation of SUMOylated STAT5, which alters its transcriptional activity in early B and T cells.

To further confirm that SUMOylated STAT5 can be regulated by SENP1, we SUMOylated STAT5 by cotransfecting FLAG-tagged STAT5A or STAT5B with HA-tagged SUMO-2 constructs in COS-1 cells. In the presence of HA-tagged SUMO-2 construct, two major bands migrating at 115 kDa and 135 kDa were detected (Figures 22A and 22B). In COS-1 cells, PIAS3 greatly enhanced STAT5 SUMOylation in a RING-domain-dependent manner (Figure 23). Notably, overexpression of SENP1 completely abrogated SUMOylated STAT5, while a SENP1 catalytic mutant (C603A mutation) could not deconjugate SUMOylated STAT5 (Figure 24). Together, SENP1 plays a critical role in regulating the SUMOylation state of STAT5 *in vivo*.

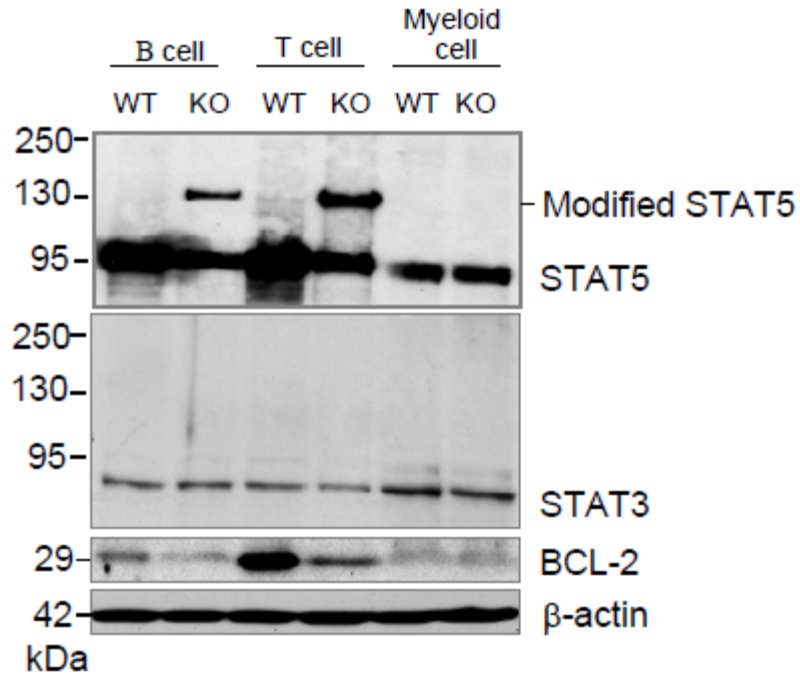


**Figure 18. The expression of key transcription factors involved in lymphoid differentiation.** RNA was isolated from fetal liver cells of  $SEN1^{+/+}$  and  $SEN1^{-/-}$  embryos at E14.5. RT-PCR was analyzed after normalization with b-actin. The results shown are a representative of at least three independent experiments.

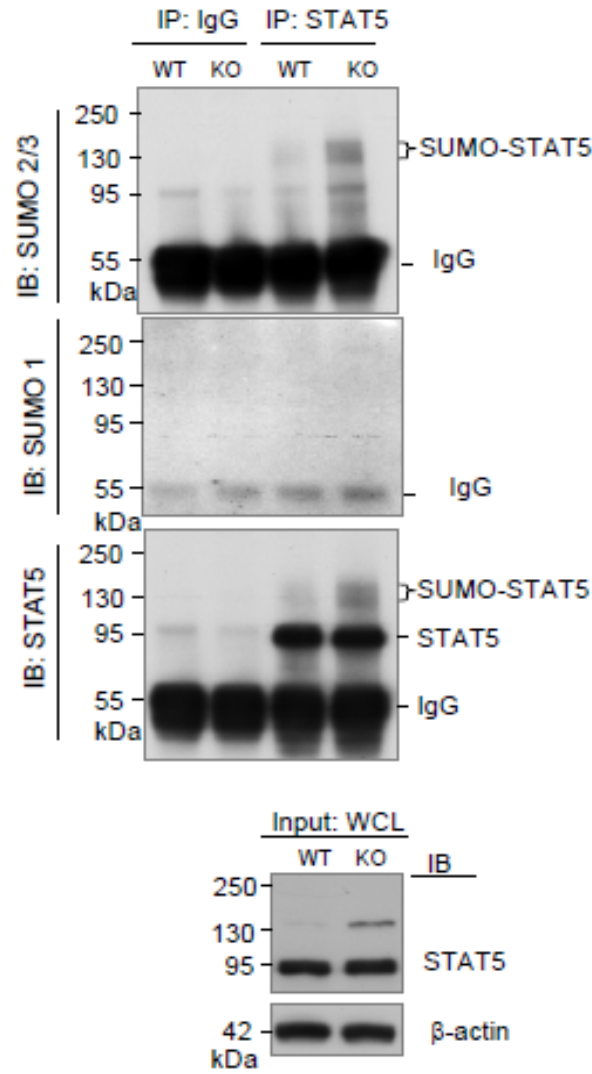


**Figure 19. The expression of IL-7R components.** RNA was isolated from fetal liver cells of  $SEN1^{+/+}$  and  $SEN1^{-/-}$  embryos at E14.5. Quantitative RT-PCR was done using TagMan assay kit from ABI. Gene expression was normalized to 18s rRNA level. The relative abundance is displayed as an average of triplicates of quantitative PCR in each sample, and error bars indicate  $\pm$  SD. The results shown are a representative of at least three independent experiments.

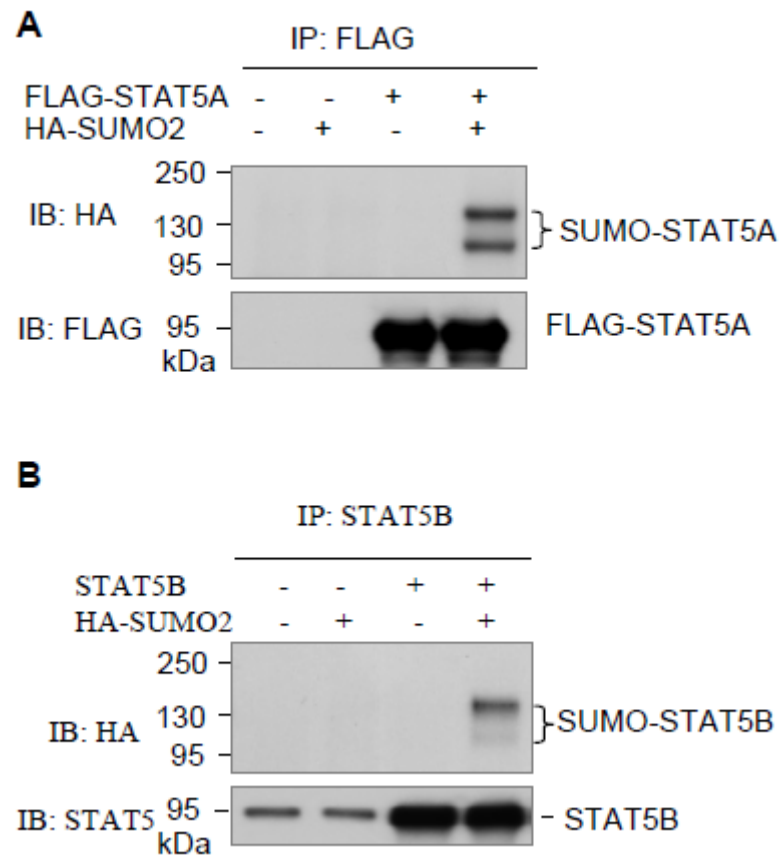




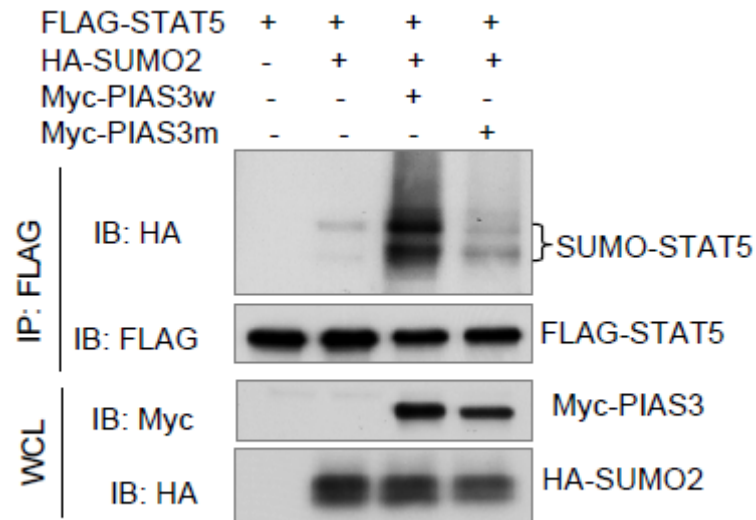
**Figure 20. Modified STAT5 accumulated in *SEN1<sup>-/-</sup>* B and T cells.** Sorted FL-HSCs from wild-type and *SEN1<sup>-/-</sup>* embryos at E14.5 were cocultured on OP9 and OP9-DL1 cells in the presence of IL-7 and Flt3L to differentiate into B and T cells, respectively. After 7 days of coculture, B and T cells were isolated and lysed in RIPA buffer. The whole cell lysates were analyzed by immunoblotting with anti-STAT5 (top panel), anti-STAT3 (second panel), anti-BCL-2 (third panel), and anti-β-actin (bottom) antibodies. Wild-type and *SEN1<sup>-/-</sup>* myeloid cells derived from HSC differentiation on OP9 stromal cells in the presence of IL3 (10ng/ml), IL6 (10ng/ml), SCF (50 ng/ml), and Flt3L (50 ng/ml) for 7 days were used as controls. The results shown are a representative of at least three independent experiments.



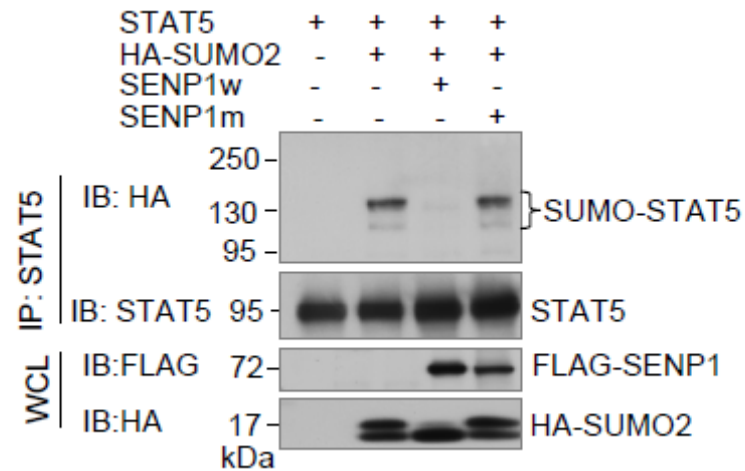
**Figure 21. Endogenous STAT5 is SUMOylated by endogenous SUMO2/3 *in vivo*.** B cells isolated from differentiation of wild-type and SENP1<sup>-/-</sup> FL-HSCs after 7 days of coculture on OP9 cells were lysed and boiled in denaturing lysis buffer. Cell lysates were aliquoted equally for performing immunoprecipitation (IP) by control IgG or a mixture of anti-STAT5A and B antibodies. Bound proteins were detected by immunoblotting (IB) with anti-SUMO2/3 (top panel), anti-SUMO1 (middle panel) or anti-STAT5 (bottom panel) antibodies. Loading samples were immunoblotted with anti-STAT5 or anti-β actin antibodies. The results shown are a representative of at least three independent experiments.



**Figure 22. Both STAT5A and STAT5B are SUMOylated in an overexpression system.** COS-1 cells were transfected with FLAG-tagged STAT5A (A) or STAT5B (B) and HA-tagged SUMO-2 plasmids. Twenty four hours after transfection, cellular protein extracts were immunoprecipitated with anti-FLAG or anti-STAT5B antibodies followed by Western blot analysis with anti-HA (top) or anti-FLAG and STAT5 B antibodies (bottom). The results shown are a representative of at least three independent experiments.



**Figure 23. SUMOylation of STAT5 is enhanced by PIAS3.** COS-1 cells were transfected with 1 mg FLAG-STAT5 + 0.5 mg HA-SUMO2 + 0.5 mg Myc-PIAS3wt or Myc-PIAS3 mut (mutant ring domain) plasmids. Twenty-four hours after transfection, transfected cells were lysed and boiled in denaturing lysis buffer for 5 min. Protein extracts were immunoprecipitated with anti-FLAG followed by Western blot analysis with anti-HA or anti-FLAG antibodies. The input was immunoblotted with anti-Myc and anti-HA antibodies. The results shown are a representative of at least three independent experiments.



**Figure 24. SENP1 de-SUMOylates SUMOylated STAT5 *in vivo*.** COS-1 cells were transfected with the indicated plasmids. Protein extracts were precipitated with anti-STAT5B antibody and analyzed with HA (top) and STAT5 (bottom) antibodies. The results shown are a representative of at least three independent experiments.

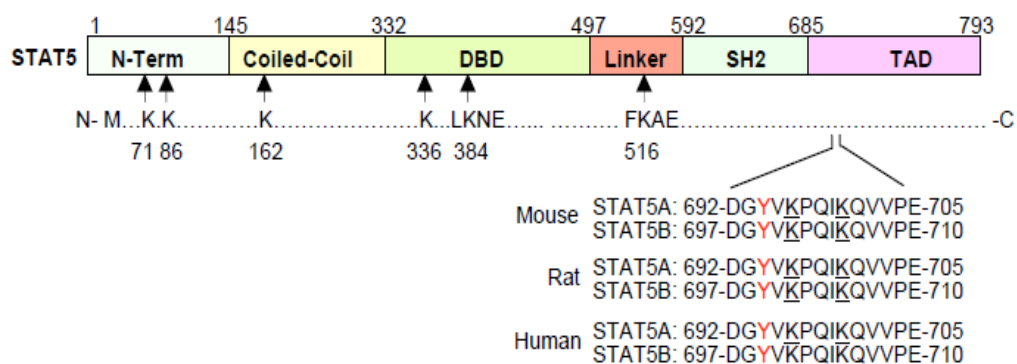
## SUMOylation inhibits tyrosine phosphorylation of STAT5

SUMOylation often, but not always, occurs on lysine residues found in the consensus motif  $\Psi$ KXE (where  $\Psi$  is any large hydrophobic residue, and X is any residue). STAT5 has two SUMO consensus motifs at lysines 384 and 516, and four high-scoring non-consensus motifs at lysines 71, 86, 163 and 336 (Figure 25). We examined the ability of these sites to be SUMOylated *in vivo* by introducing single or combined mutations (six lysine-to-arginine mutations). Compared with SUMOylation of wild-type STAT5, SUMOylation of single mutants or combined mutants remained unchanged, suggesting that these lysine residues are not involved in SUMOylation of STAT5 (Figure 26). Previous studies indicate that STAT1 is SUMOylated at lysine 703, close to tyrosine 701 (64, 110, 111). Indeed, STAT5A contains two lysines at 696 and 700, close to tyrosine 694 (Figure 25). These lysine residues of STAT5A/B are well conserved across species including mouse, rat and human. SUMOylation of single mutants (K696R and K700R) or double mutant (K696/700R) of STAT5A was drastically reduced (Figure 27A). These results indicate that lysine residues 696 and 700 are the major sites for SUMOylation of STAT5A *in vivo*. Consistently, the major SUMOylation sites in STAT5B were lysine residues 701 and 705 (Figure 27B).

Since the SUMOylation sites are in close proximity to tyrosine, whose phosphorylation is a prerequisite for STAT5 activation, we therefore assessed the interplay between STAT5 phosphorylation and SUMOylation. We checked the level of STAT5 phosphorylation in *SENP1*<sup>-/-</sup> B and T cells derived from HSC

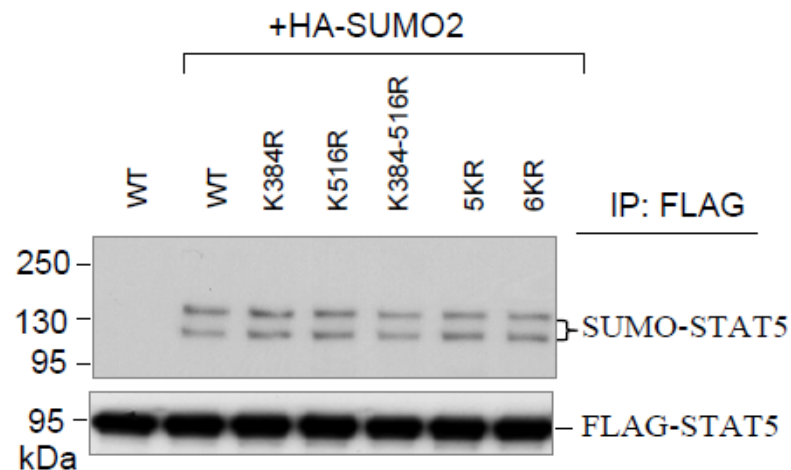
differentiation *in vitro* after 7 days of coculture on OP9 and OP9-DL1 cells in the presence of IL-7 and Flt3L, respectively. Western blot analysis showed a marked reduction of STAT5 phosphorylation in SENP1<sup>-/-</sup> B and T cells (Figure 23), correlating with an increased SUMOylation (Figure 20). Notably, the phosphorylation of STAT5 SUMOylated form could not be detected in SENP1<sup>-/-</sup> B and T cells. These results suggest that in the absence of SENP1, SUMOylation inhibits STAT5 phosphorylation and that the accumulation of SUMOylated STAT5 impairs the activation–inactivation cycle of STAT5 in lymphocytes.

We further tested the effect of phosphorylation at tyrosine 694 on SUMOylation of STAT5. Wild-type STAT5 and constitutively active STAT5 mutant N642H were efficiently SUMOylated. In contrast, STAT5 phosphorylation mutant Y694A abolished SUMOylation of STAT5 (Figure 29). These results strongly suggest that SUMOylation of STAT5 requires a prior phosphorylation or nuclear import event since the enzymes involved in SUMOylation are located in the nucleus.

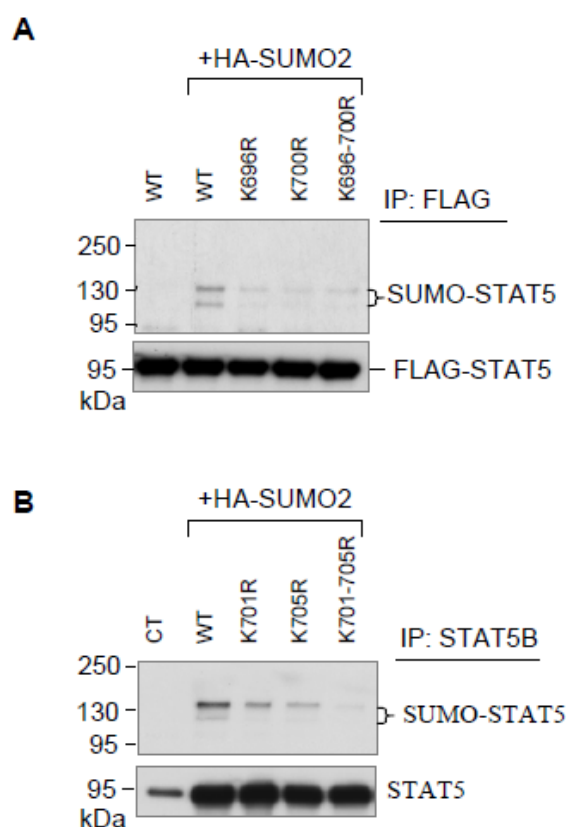


**Figure 25. Schematic diagram of full-length murine STAT5 protein structure.** STAT5 contains amino terminal, coiled-coil, DNA binding, linker, SH2, and transcriptional activation domains. Arrows indicate approximate locations of two putative SUMO consensus motifs at lysines 384 and 516 ( $\Psi$ -K-X-E, where  $\Psi$  represents a large hydrophobic amino acid (A, F, I, L, M, V, W) and X represents any amino acid), and four other non-consensus motifs at lysines 71, 86, 163 and 336. Two lysine residues 696 and 700 located close to Y694 and Y699 of STAT5A and STAT5B respectively are underlined.

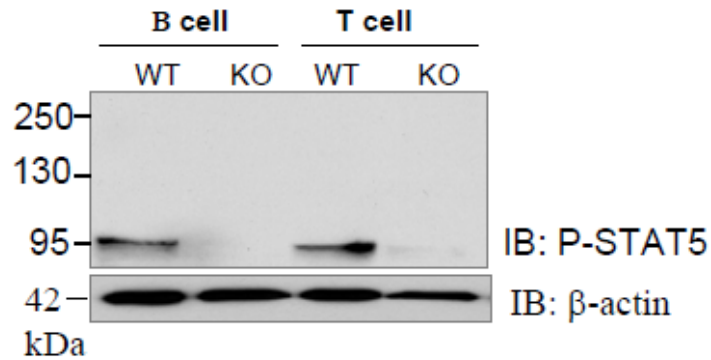




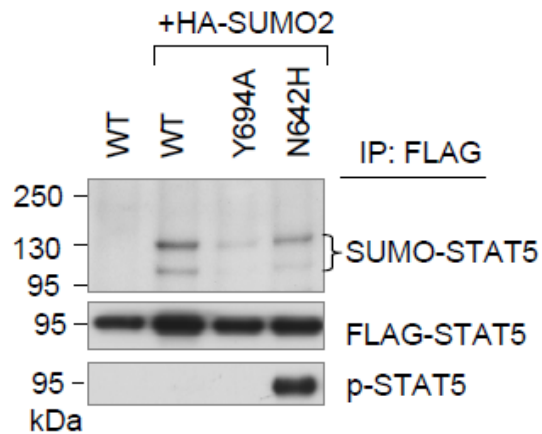
**Figure 26. STAT5 is not SUMOylated at lysine residues within the SUMO consensus motifs.** COS-1 cells were transfected with FLAG-tagged STAT5A and its mutants along with HA-SUMO2 plasmids. Protein extracts were immunoprecipitated with anti-FLAG and then revealed by Western blot analysis with antibodies to anti-HA (top) and anti-FLAG (bottom). 5KR: 5 lysine-to-arginine mutations at positions 71, 86, 336, 384, and 516. 6KR: 6 lysine-to-arginine mutations at positions 71, 86, 163, 336, 384, and 516. The results shown are a representative of at least three independent experiments.



**Figure 27. mapping STAT5 SUMOylation sites.** (A) STAT5A is modified by SUMO at lysine residues 696 and 700. COS-1 cells were transfected with FLAG-tagged STAT5A and its mutants along with HA-SUMO2 plasmids. Protein extracts were immunoprecipitated with anti-FLAG and then revealed by Western blot analysis with antibodies to anti-HA (top) and anti-FLAG (bottom). The results shown are representative of at least three independent experiments. (B) Identification of SUMOylation sites in STAT5B. COS-1 cells were transfected with STAT5B and its mutants along with HA-SUMO2 plasmids. Immunoprecipitates of STAT5B were immunoblotted with anti-HA (top) or anti-STAT5B antibodies (bottom). The results shown are a representative of at least two independent experiments.



**Figure 28. Tyrosine phosphorylation of STAT5 is diminished in  $SEN1^{-/-}$  B and T cells.** Sorted FL-HSCs from wild-type and  $SEN1^{-/-}$  embryos at E14.5 were cocultured on OP9 and OP9-DL1 cells in the presence of IL-7 and Flt3L to differentiate into B and T cells, respectively. After 7 days of coculture, B and T cells were isolated and lysed in RIPA buffer. The whole cell lysates (prepared and analyzed in Fig. 3A) were further analyzed by immunoblotting with anti-P-STAT5 (top panel) and anti- $\beta$ -actin (bottom) antibodies. The results shown are a representative of at least three independent experiments.



**Figure 29. SUMOylation of STAT5 is tyrosine phosphorylation-dependent.** COS-1 cells were transfected with FLAG-tagged STAT5A (wild-type), STAT5 phosphorylation mutant Y694A (mutation of tyrosine 694 to alanine) and constitutively active STAT5 mutant N642H, and HA-tagged SUMO-2 plasmids. Twenty four hours after transfection, Cellular protein extracts were immunoprecipitated with anti-FLAG antibody followed by Western blot analysis with anti-HA (top) or anti-FLAG antibodies (bottom). The results are a representative of at least three independent experiments.

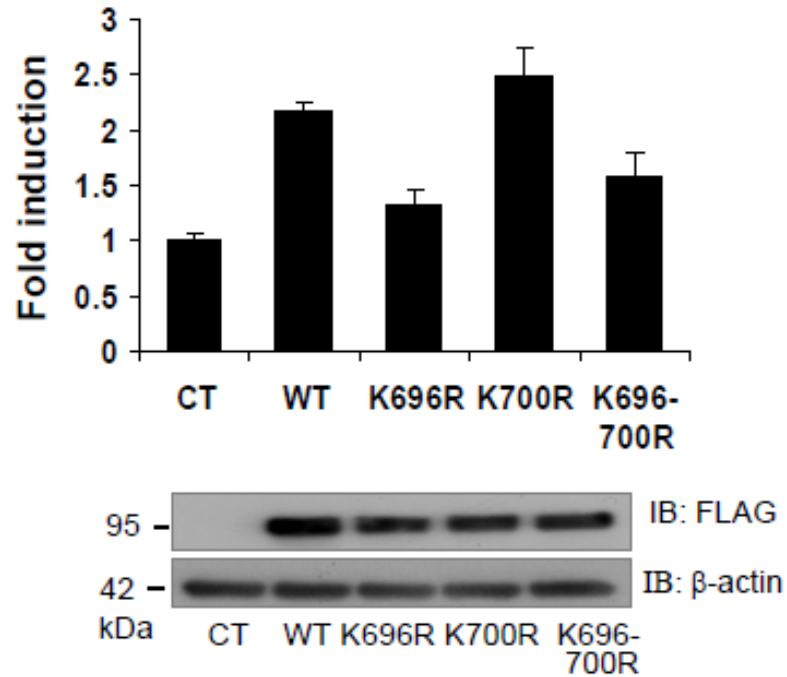
### **SENP1 controls an acetylation/SUMOylation switch in STAT5**

To further understand the effect of SUMOylation on STAT5-mediated transcriptional activity, we compared wild-type and SUMOylation-deficient STAT5A for their ability to activate transcription in reporter assays. STAT5-deficient MEF cells stably expressing wild-type STAT5A and its mutants were transiently transfected with a luciferase reporter pGL4- $\beta$ -Casein. Compared with wild-type STAT5A, STAT5A(K700R) exhibited a similar transactivation activity, suggesting that SUMOylation of STAT5A at lysine 700 did not play a role in transcription regulation (Figure 30). However, STAT5A(K696R) or STAT5A(K696-700R) showed a markedly decreased in transactivation activity, raising an intriguing possibility that lysine 696 may also be subjected to another form of post-translational modification.

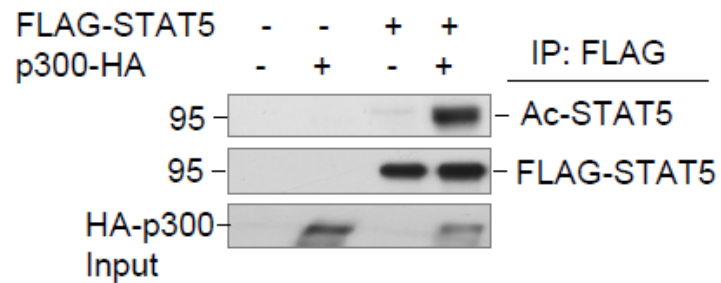
Lysine residues can be targeted by multiple modifications, such as ubiquitination, SUMOylation, methylation, and acetylation. The carboxy-terminal transactivation domain of STAT5 is known to interact with the histone acetyltransferase p300/CBP, resulting in enhanced STAT5-dependent transcription (112). We hypothesized that lysine 696 is also a target for acetylation. To test this possibility, we first checked whether STAT5 can be acetylated by p300. Coexpression of p300 in HEK-293 cells significantly enhances STAT5A acetylation (Figure 31). Wild-type STAT5A and STAT5A(K700R) mutant, but not STAT5A(K696R) and STAT5A(K696-700R) mutants, were acetylated, suggesting that lysine 696 is also a major acetylation site (Figure 32). Consistent with our data, a recent study indicates that STAT5B acetylation on lysine residues including lysine

701 (corresponding lysine 696 on STAT5A) is essential for STAT5B dimerization since mutation of lysine 701 to arginine impaired STAT5B dimerization and transcriptional activity (57).

Next, we assessed endogenous STAT5 acetylation in wild-type and SENP1<sup>-/-</sup> FL cells. Protein extracts were immunoprecipitated with anti-STAT5 antibody and analyzed by immunoblotting with antibody specific for K701-acetylated STAT5B. As shown in Figure 33, endogenous STAT5 was acetylated in both wild-type and SENP1<sup>-/-</sup> FL cells treated with IL-7. Notably, STAT5 acetylation in IL7-treated SENP1<sup>-/-</sup> FL cells was significantly decreased compared with that of IL7-treated wild-type FL cells, suggesting that SENP1 regulates the acetylation status of STAT5. To confirm the role of SENP1 in the regulation of STAT5 acetylation and SUMOylation, HEK-293 cells were transfected with FLAG-tagged STAT5A, MYC-tagged SUMO3, HA-tagged p300 in the presence of RGS-tagged SENP1 or RGS-tagged SENP1 mutant plasmids. Overexpression of SENP1 enhanced acetylation and inhibited SUMOylation of STAT5A (Figure 34). In contrast, overexpression of SENP1 mutant decreased acetylation and enhanced SUMOylation of STAT5 (Figure 34). Taken together, these results demonstrate that SENP1 plays a critical role in regulating an acetylation/SUMOylation switch in STAT5.

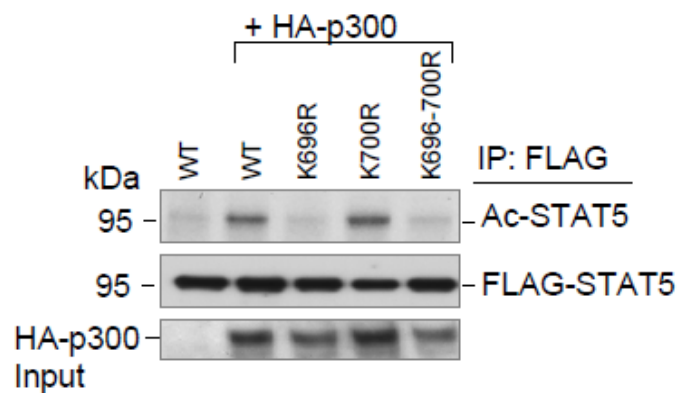


**Figure 30. The K696R mutant of STAT5 impairs its transcriptional activity.** STAT5A/B-deficient MEFs, infected with an empty lentiviral vector (control, CT) or lentiviral vectors directing expression of wild-type STAT5, STAT5K696R, STAT5K700R or STAT5K696-700R, were transiently transfected with a luciferase reporter (pGL4-b-Casein) in triplicates. After 6-hour transfection, the cells were cultured in serum-free medium in the presence or absence of GH (200 ng/ml) for 18 hours. Samples were harvested and evaluated for luciferase and renilla activity. The fold induction was calculated as the ratio of luciferase activity in the presence and absence of GH. Data shown are representative of three independent experiments. Error bars indicate standard deviation. The lower panel shows STAT5 expression levels in different clones measured by Western blot analysis with anti-FLAG and anti-β-actin (control) antibodies.

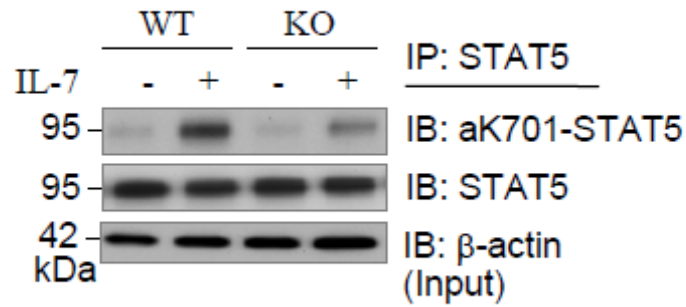


**Figure 31. STAT5 is acetylated by p300.** HEK-293 cells (in 6-well plate) were transfected with indicated FLAG-tagged STAT5A and HA-tagged p300 plasmids. Immunoprecipitation was performed with EZview Red ANTI-FLAG M2 Affinity Gel beads and analyzed with anti-acetyl lysine (top panel) and anti-FLAG (second panel) antibodies. The results are a representative of at least three independent experiments.

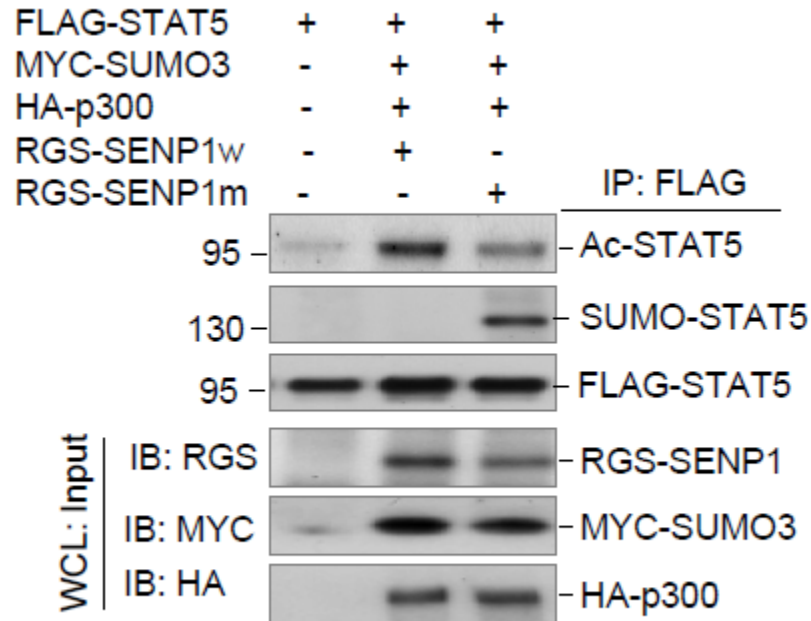




**Figure 32. Lysine 696 is also a target for acetylation.** Lysates from HEK-293 cells transfected with plasmids expressing FLAG-tagged STAT5A or SUMOylation-deficient mutants of STAT5A and HA-p300 were immunoprecipitated with EZview Red ANTI-FLAG M2 Affinity Gel beads and analyzed with the indicated antibodies. The results are representatives of at least three independent experiments.



**Figure 33. Endogenous STAT5 is acetylated in FL cells treated with IL-7.** FL cells isolated from three to five E14.5 wild-type and *SEN1<sup>-/-</sup>* embryos were treated with IL-7 for 10 min and lysed in RIPA buffer. Cellular extracts were immunoprecipitated with anti-STAT5 antibody and analyzed with anti-aK701-STAT5B (top panel) and anti-STAT5 (second panel) antibodies. Loading samples were immunoblotted with anti-β actin antibody. The results shown are a representative of three independent experiments.



**Figure 34. SENP1 regulates the SUMOylation/acetylation status of STAT5.**

Lysates from HEK-293 Cells (in 6-well plate) transfected with plasmids expressing FLAG-tagged STAT5A, MYC-tagged SUMO3, HA-tagged p300 and RGS-tagged SENP1w (wild-type) or RGS-tagged SENP1m (mutant) were immunoprecipitated with EZview Red ANTI-FLAG M2 Affinity Gel beads and analyzed with anti-acetyl lysine (top panel), anti-SUMO2/3 (second panel) and anti-FLAG (third panel) antibodies. The input was immunoblotted with anti-RGS, anti-Myc and anti-HA antibodies. The results shown are a representative of at least three independent experiments.

## DISCUSSION

### **SEN1 is essential for early lymphoid development**

SUMOylation has emerged as an important regulatory mechanism for protein function (13, 19). SENPs are essential for reversing SUMOylation in many different systems, but the physiological functions of SENPs are poorly understood. Recently, SENP1 has been shown to play a crucial role in regulating erythropoiesis (20-22). However, the physiological function of SENP1 in the development of the immune system remains unknown. In the present study, we have discovered that SENP1 is essential for early T and B lymphopoiesis.

The IL-7/IL-7R signaling pathway plays a critical role in regulating T and B cell development by controlling several cellular processes, such as cell survival, proliferation and VDJ recombination through alteration of chromatin structure. Loss of IL-7R $\alpha$ ,  $\gamma$ C or Jak3 gene in mice leads to a severe developmental block of T and B cells (113-117), and mutations of these genes results in severe combined immunodeficiency in humans (118-121). IL-7 signaling is required for early T cell development in the thymus, mainly to protect cells at the DN 2 and DN3 stages from apoptotic cell death by regulating the expression of the survival proteins Bcl-2 and Mcl-1. Two groups independently crossed IL-7R-deficient mice to mice expressing a *Bcl2* transgene. As a result, enforced expression of BCL-2 was sufficient to partially restore T cell development but was unable to rescue B cell development in IL-7R-deficient mice (122-124). These studies suggested that the IL-7R signaling not only promote pro-B cell survival, but also regulates the expression of other genes required

for B cell development. Consistent with this notion, a recent study indicates that IL-7R signaling is required for stage transition in adult B cell development through up-regulation of the early B cell transcription factor EBF (125).

The transcription factor STAT5 is a key molecule downstream of The IL-7/IL-7R signaling pathway. Earlier analysis of *STAT5* knockout mice (*STAT5AB*<sup>ΔN/ΔN</sup> mice), which express N-terminally truncated and partially functional STAT5 proteins, revealed only a mild defect in T and B cell development (126-128). However, a complete inactivation of STAT5A/B in mice (*STAT5AB*<sup>null/null</sup> mice) exhibited a profound defect in early T and B cell development (107-109). These studies clearly indicate the importance of STAT5 in regulating early T and B cell development. Our data indicate that SENP1 deficiency results in severe defects in early T and B cell development, similar to that observed in *STAT5AB*<sup>null/null</sup> mice, suggesting that STAT5 may be a direct target of SENP1. In agreement with this hypothesis, we also found that BCL-2, a key downstream target of STAT5 is significantly downregulated in SENP1<sup>-/-</sup> T and B lymphocytes.

A possible explanation for the impaired development of both T and B cells observed in SENP1<sup>-/-</sup> embryos could be decreased numbers or function of HSCs. However, we have previously reported that HSCs were not affected in SENP1<sup>-/-</sup> embryos (21). Consistent with our previous findings, myeloid development is not affected in SENP1 deficiency. We also observed that SENP1 is expressed very high in HSCs (Figure 6). This suggests that SENP1 may be required for long-term self-renewal capacity of HSCs. Future studies are needed to address this important issue.

### **STAT5 is regulated by the SUMOylation pathway**

PIAS proteins were initially identified as negative regulators of STAT signaling that inhibit the activity of STAT-transcription factors (58, 59). The exact molecular mechanisms by which the PIAS proteins inhibit STAT activity remain unclear, although several models have been proposed. For example, PIAS proteins bind only to activated STAT dimmers in the nucleus and inhibit their DNA-binding activity. Other possible explanation for the role of PIAS proteins in the regulation of STAT activity is that PIAS protein can recruit other co-regulators, including histone deacetylases (HDACs), to repress transcription. A recent study reported that PIAS1 binding to the *Foxp3* promoter leads to recruitment of DNA methyltransferases and heterochromatin protein 1 for epigenetic modifications (61). *Pias1* deficiency results in promoter demethylation, reduced histone H3 methylation at Lys9, and enhanced promoter accessibility. Interestingly, deletion of PIAS1 leads to the enhanced binding of STAT5, a key transcription factor involved in *Foxp3* induction to the *Foxp3* promoter. As a result, *PIAS1*<sup>-/-</sup> mice exhibit the increased frequency of *Foxp3*<sup>+</sup> CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (61). In deed, SUMOylation of many transcription factors results in alterations of their localization or interaction with binding partners. In most cases, SUMOylated proteins suppress transcription, possibly due to their interaction with co-repressors. Thus, the mechanism that SUMOylation regulates transcription is quite similar to that a PIAS protein suppresses the transcriptional activity of transcription factors. It is likely that PIAS proteins regulate the transcriptional activity of transcription factors through the SUMOylation pathway.

PIAS proteins have SUMO-specific E3 ligase activity, and have been shown to promote the SUMOylation of numerous transcription factors, such as p53, LEF1 and c-Jun (129-131). These studies raised the possibility that STAT activity might be regulated by the SUMOylation pathway. It was previously shown that STAT1 are SUMOylated on Lysine 703 in an overexpression system (63, 64, 111). However, the *in vivo* function of SUMOylation in the regulation of STAT1 activity remains unclear. In addition, it is not known whether other STATs can be modified by SUMO. In this study, we found that SUMOylation negatively regulates STAT5 activity. The accumulation of SUMOylated STAT5 results in suppression of STAT5 activity and subsequent signaling events in SENP1<sup>-/-</sup> T and B cells. Although we also found that STAT5 SUMOylation is greatly enhanced by PIAS3 in an overexpression system, further studies will be required to define whether this SUMO-specific E3 ligase is involved in regulation of STAT5 activity *in vivo*.

### **SENP1 regulate STAT5 SUMOylation**

Protein function is tightly regulated by reversible posttranslational modifications to create an on and off state that is crucial for many biological processes. Many proteins are dynamically modified at multiple sites by different modifications (26). The histone code hypothesis has been proposed that distinct histone modifications, on one or more tails, act alone, sequentially or in combination to form a 'histone code' that is then read by effector proteins to bring about distinct downstream events (132). On the histone H3 tail, lysines 9 and 27 are well-known methylation sites, and methylation of these sites is 'read' by heterochromatin protein

1 (HP1) and Polycomb (Pc), leading to heterochromatin-mediated gene silencing (Lys 9) and epigenetic repression (Lys 27), respectively (133, 134). It is noteworthy that lysines 9 and 27 are located the “RK9S” or “RK27S” motif. According to the “methyl/phos switch” model, serine phosphorylation of the “RKS” motif that engages an HP1 or Pc module could lead to consecutive loss of binding (44).

The interplay between phosphorylation and SUMOylation of neighboring sites has been shown to play an important role in regulating the transcriptional activity of many transcription factors. For example, heat-shock factors (HSFs), GATA-1 and myocyte enhancer factor 2 (MEF2), containing a SUMO consensus site and an adjacent proline-directed phosphorylation site ( $\Psi$ KxE<sub>xx</sub>SP), are regulated by phosphorylation-dependent SUMOylation (27, 28, 135). The motif  $\Psi$ KxE<sub>xx</sub>SP couples sequential phosphorylation and SUMOylation and has been referred to as a “Phospho-SUMOyl switch” (26). It has been shown that STAT1 SUMOylation at lysine 703 inhibits its phosphorylation at tyrosine 701 (136). In addition, STAT1 SUMOylation at lysine 703 and STAT1 phosphorylation at tyrosine 701 are mutually exclusive (137). We have identified two SUMOylation sites of STAT5 at lysines 696 and 700, located in close proximity to tyrosine 694, whose phosphorylation is a prerequisite for STAT5 activation, suggesting a possible interplay between SUMOylation and phosphorylation in regulating STAT5 activity. Indeed, SUMOylation of STAT5 is phosphorylation-dependent, since STAT5 phosphorylation mutant Y694A abolished SUMOylation of STAT5 (data not shown). We also found that SENP1 deficiency causes increased STAT5 SUMOylation, correlating with diminished STAT5 phosphorylation and activity in



lymphocytes (Figures 20 and 28), suggesting that SUMOylation of STAT5 inhibits its phosphorylation and subsequent signaling events. Interestingly, SUMOylation of STAT5 observed in the absence of SENP1 appears to be lymphocyte specific, since no SUMOylated STAT5 could be detected in SENP1<sup>-/-</sup> myeloid cells (Figure 20). Further investigations are required to resolve this issue.

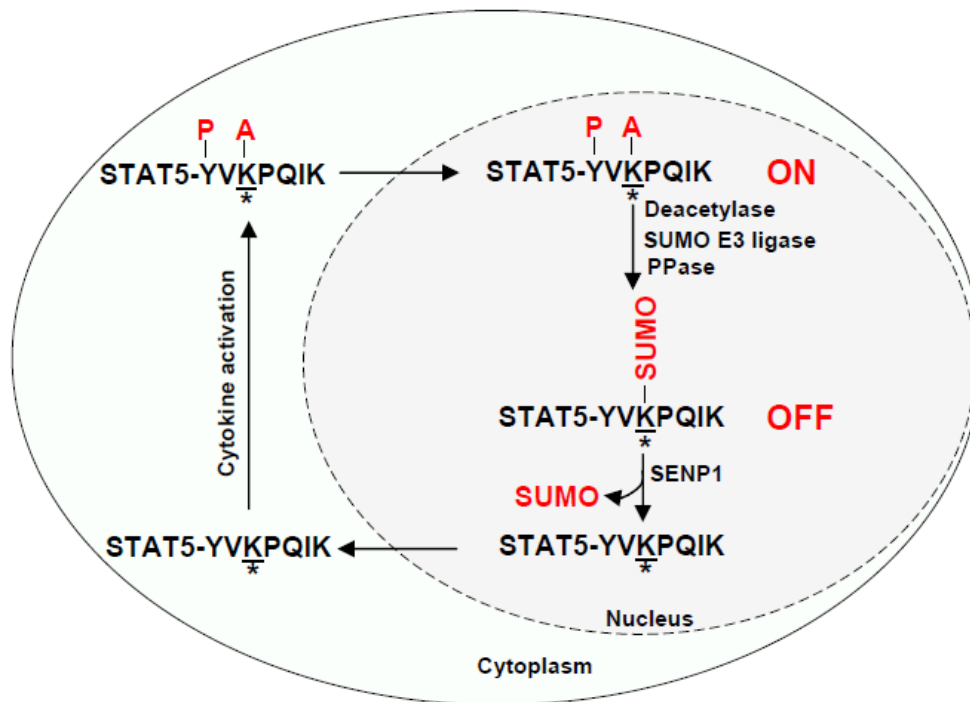
### **The acetylation/SUMOylation switch in STAT5 is regulated by SENP1**

Since lysine can be a target of different posttranslational modifications, SUMOylation can block alternative lysine-targeted modifications, such as ubiquitination, methylation or acetylation. It was previously reported that IκBα is SUMOylated on K21, which is also targeted by ubiquitination (29). Transcriptional activity of several transcription factors, such as SP3, HIC1 and MEF2A can be regulated by interplay between SUMOylation and acetylation on the same lysine residue (30, 31, 138). An acetylation-SUMOylation switch on MEF2 is further regulated by its phosphorylation (31). In addition to tyrosine phosphorylation, acetylation of different STATs has been shown to play a critical role in regulating their activity (52, 53, 55, 56). For example, STAT3 acetylation at lysine 685 is essential for its dimerization and transcriptional activity (55). Here, our data clearly show that STAT5A is acetylated at lysine 696, which is also a target for SUMOylation. Acetylation of STAT5 at lysine 696 is essential for STAT5 activation, since mutation of this lysine diminished the transcriptional activity of STAT5 (Figure 30). Consistent with our finding, a recent study has reported that STAT5B acetylation on lysine 701 (corresponding lysine 696 on STAT5A) is

essential for STAT5B dimerization and transcription (57). Notably, our data provide direct evidence that SENP1 regulates an acetylation/SUMOylation switch in STAT5.

### **Proposed model**

Based on the findings reported here, we propose a model for the role of SENP1 in the regulation of an acetylation-SUMOylation switch in STAT5 (Figure 35). In unstimulated cells, STAT5 exists in the cytoplasm as a monomer. Upon activation, tyrosine-phosphorylated and acetylated STAT5 dimerizes, translocates to the nucleus, and activates transcription. We currently do not know which signal induces the acetylation/SUMOylation switch in STAT5. Since we did not observe the phosphorylation of STAT5 SUMOylated form in SENP1<sup>-/-</sup> B and T cells (Figure 20), it seems likely that dephosphorylation of STAT5 promotes a switch from acetylation (active state) to SUMOylation (inactive state). It is also possible that SUMOylation of STAT5 facilitates its dephosphorylation. SENP1 protein, which is predominantly present in the nucleus (16), is required for de-conjugating SUMOylated STAT5 before it returns to the cytoplasm to complete an activation-inactivation cycle. In the absence of SENP1, STAT5 is accumulated in the SUMOylation state, leading to inhibition of STAT5 phosphorylation and acetylation, and subsequent signaling events. Thus, our findings establish a specific role of SENP1 in regulating lymphoid development via an acetylation/SUMOylation switch in STAT5.



**Figure 35. A model for the role of SENP1 in the regulation of acetylation-SUMOylation switch in STAT5.** SUMOylation switches STAT5 from an active state to an inactive state. SENP1 is required to remove SUMO from conjugated STAT5, allowing it to re-enter the activation-inactivation cycle. \* is lysine 696 in STAT5A or lysine 701 in STAT5B; A is acetylation; P is phosphorylation.

In conclusion, our study has provided evidence that SENP1 controls early T and B lymphopoiesis through the regulation of an acetylation/SUMOylation switch in STAT5. SENP1 deficiency results in severe defects in early T and B cell development. Our data clearly demonstrate that SENP1 regulates STAT5 activity via an acetylation/SUMOylation switch in STAT5. SENP1 deficiency causes the accumulation of SUMOylated STAT5, thus preventing STAT5 to re-enter another activation-inactivation cycle, which contributes to defective early T and B cell development. The acetylation/SUMOylation switch may act as an important mechanism involving in the regulation of transcription in the immune system to turn the signaling ON and OFF in response to cytokines or pathogens.

## MATERIALS AND METHODS

### Mice

The generation and screening of  $SEN1^{-/-}$  fetuses has been described previously (21).  $SEN1^{+/-}$  mice were intercrossed, and E14.5 fetuses (date of plug = E0.5) were obtained for FL isolation.  $RAG1^{-/-}$  mice were obtained from The Jackson Laboratory. All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee at The University Texas M. D. Anderson Cancer Center.

### Plasmids

The full-length cDNA of murine STAT5A was amplified from the retroviral vector pMIG-STAT5 (a gift of Drs. Yong-Jun Liu & Stephanie S. Watowich, UT-MD Anderson cancer center), and then subcloned into p3xFLAG-CMV<sup>TM</sup>-7.1 Expression Vector (Sigma, St Louis, MO) using standard techniques. Rat STAT5b pcDNA3.1- was a kind gift from Dr. L.-Y. Yu-Lee (Baylor College of Medicine). Myc-tagged wild-type mouse PIAS3 and Myc-tagged mouse PIAS3 RING domain mutant were generously provided by Dr. Fang Liu (Rutgers University). pGL4 mouse  $\beta$ -Casein reporter plasmid was kindly given by Dr. Koichi Ikuta (Kyoto University). pGL4.74 [hRluc/TK] vector was from Promega. HA-p300 plasmid was described earlier (139). HA-SUMO2, Flag-SEN1, and Flag-SEN1 catalytic mutant plasmids were generated in our laboratory. STAT5 mutants were generated by using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

## **Antibodies and Cytokines**

Anti-Flag (M2, F3165) was from Sigma. Anti-HA (influenza hemagglutinin) (16B12, MMS-101P) was from Covance. Anti-RGS (34610) was from QIAGEN. Anti-Myc (9E10) was from Santa Cruz Biotechnology. Monoclonal anti-SUMO-1 antibody (anti-GMP-1) was from Zymed. Anti-SUMO-2/3 was purchased from Abgent. Anti-STAT5 (C-17) and anti-STAT3 (C-20) antibodies were from Santa Cruz Biotechnology. Antibodies for STAT5A and STAT5B were from R&D Systems. Anti-phospho-STAT5 was from cell Signaling. Anti-Bcl-2 antibody (610538) was from BD Biosciences. Anti-acetylated-lysine antibody (9441s) was from Cell Signaling. Anti-aK701-STAT5b was from Dr. Y. Eugene Chin (Brown University) and described previously (57). Anti- $\beta$ -Actin was from Sigma.

All cytokines, including murine IL-7, murine Ilt3 ligand, murine IL-3, murine IL-6 and murine SCF, were purchased from Peprotech (Rocky Hill, NJ).

## **Monoclonal antibodies and flow cytometry**

All monoclonal antibodies used in this study were from BD Pharmingen: anti-c-Kit (2B8; APC), anti-Sca-1 (E13-161.7; PE and D7; FITC), anti-TER-119 (TER-119; FITC, APC, PE), anti-CD11b (M1/70; FITC), anti-Gr-1 (RB6-8C5; PerCP-Cy5.5), anti-CD19 (1D3; PerCP-Cy5.5), anti-CD4 (RM4-5; PerCP-Cy5.5 and GK1.5; FITC), anti-CD8a (53-6.7; PerCP-Cy5.5, APC), anti-CD44 (Pgp-1; FITC, IM7; APC), anti-CD25 (7D4; FITC, PE), anti-mouse early B lineage (AA4.1; FITC), anti-CD24 (M1/69; FITC), anti-BP1 (6C3; PE, biotin), anti-CD43 (S7; FITC, biotin),

anti-B220 (RA3-6B2; APC, PE), anti-IgM (II/41; APC). For detection biotinated antibodies, streptavidin-APC and PerCP-Cy5.5 (BD Pharmingen) were used.

Single-cell suspensions were first incubated on ice with rat anti-mouse CD16/CD32 (BD PharMingen) to block nonspecific binding to Fc receptors. Subsequently, cells were incubated with indicated antibodies and analyzed using a LSR II flow cytometer or FACS-Calibur machine and the FlowJo analyzing software (Treestar).

### **Purification of FL-HSCs**

E14.5 fetal liver cells were harvested and treated with red blood cell lysis buffer (Sigma). Then, cells were stained with anti-c-Kit-APC and anti-Sca-1-PE antibodies, and a mixture of antibodies to lineage markers: Gr-1, CD11b, TER-119, CD19, CD4 and CD8. FL-HSCs (lin<sup>-</sup> c-Kit<sup>high</sup> Sca-1<sup>high</sup>) were sorted using FACS Aria cell sorter (BD Biosciences).

### **B and T cell differentiation *in vitro***

OP9 and OP9-DL1 stromal cell lines (generous gifts from Dr. Juan Carlos Zúñiga-Pflücker, University of Toronto, Toronto, Canada) were co-cultured as previously described (104). Briefly, stromal cells were maintained in alpha-MEM (Gibco BRL) containing 20% FBS (Gibco BRL) and penicillin–streptomycin (Sigma-Aldrich). Wild-type and SENP1<sup>-/-</sup> FL-HSCs (1000-3000 cells/well in 24-well plates) were placed on OP9 and OP9-DL1 monolayers, for B and T lineage differentiation, respectively. Co-culture media contained: alpha-MEM, 10% FBS, 10

mM HEPES (Gibco BRL), 1 mM sodium pyruvate (Gibco BRL), 2 mM GlutaMax, penicillin–streptomycin, 50 mM 2-mercaptoethanol, 5 ng/ml IL-7 and 5 ng/ml Flt3L. After 4 days of culture, the cells were passed onto newly prepared OP9 and OP9-DL1 cells. For myeloid differentiation, wild-type and SENP1<sup>-/-</sup> FL-HSCs were placed on OP9 monolayers in the presence of IL3 (10ng/ml), IL6 (10ng/ml), SCF (50 ng/ml) and Flt3L (50 ng/ml).

### **Transplantation experiments**

Two x 10<sup>6</sup> fetal liver cells isolated from E14.5 wild-type and SENP1<sup>-/-</sup> embryos were injected intravenously into lethally (950 rad) irradiated RAG-1-deficient mice. Mice were sacrificed 5-6 weeks after transplantation. The lymphoid compartments of the recipients were analyzed by flow cytometry. The experiments shown in this study represent results from two independent experiments.

### **RNA Extraction and real time PCR assay**

Total RNA was extracted using the RNeasy Mini Kit from QIAGEN and converted into cDNA using Advantage RT-for-PCR Kit (Clontech) according to the protocols described in the handbooks. Quantitative RT-PCR were performed using TagMan gene expression assay from (Applied Biosystems) and analyzed on the GeneAmp 7700 sequence detection system (Applied Biosystems). Gene expression was normalized to 18s rRNA level. The relative abundance is displayed as an average of triplicates of quantitative PCR in each sample, and error bars indicate  $\pm$  SD. All primers were purchased from Applied Biosystems: mouse SENP1



(Mm00522968\_m1), mouse interleukin 7 receptor (Mm00434295\_m1), mouse Janus kinase 3 (Mm00439962\_m1), mouse Janus kinase 1(Mm00600614\_m1), mouse interleukin 2 receptor gamma chain (Mm00442885\_m1), eukaryotic18s rRNA (4319413E).

RT-PCR was performed using gene specific primers: PU1, E2A and GATA3 (140). The primers for STAT5 and  $\beta$ -Actin were described previously (21).

### **Luciferase assays**

STAT5 and its mutants were constructed in pCDH-T2AcGFP-MSCV (System Biosciences). The lentiviruses were generated according to the manufacturer's protocol (System Biosciences). STAT5A/B-deficient MEFs (a kind gift from Dr. James N. Ihle at the St. Judes Childrens Research Hospital) infected with an empty lentiviral vector (control) or lentiviral vectors directing expression of wild-type STAT5A and its mutants including STAT5K696R, STAT5K700R or STAT5K696-700R, were transiently transfected with luciferase reporter (pGL4- $\beta$ -Casein) and pGL4.74 [hRluc/TK] plasmids in triplicate using FuGENE HD (Roche). After 6-hour transfection, the cells were cultured in serum-free medium in the presence or absence of GH (200 ng/ml) for 18 hours. Samples were harvested and evaluated for luciferase and renilla activity. The fold induction was calculated as the ratio of luciferase activity in the presence and absence of GH. Data are representative of three independent experiments. Error bars indicate standard deviation.

### **Western blot analysis**

B, T and myeloid cells derived from HSC differentiation on day 7 of coculture with OP9 cells and OP9-DL1 cells were harvested and filtered through a 70- $\mu$ m filter to exclude pieces of disrupted monolayer. After washing twice with ice-cold PBS, the cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, 0.1 % SDS, [pH 7.5]) supplemented with complete protease inhibitor cocktail and 20 mM N-Ethylmaleimide (NEM, Sigma). Whole-cell protein extracts were prepared and quantified by the Bradford method (Bio-Rad, Hercules, CA). Equal amounts of protein (20  $\mu$ g/lane) was electrophoretically separated on SDS-PAGE and transferred to a PVDF membrane. The membranes were blotted with indicated antibodies. An anti-rabbit or anti-mouse antibody conjugated to horseradish peroxidase was used as secondary antibodies, and the signal was detected using a chemiluminescence kit (PerkinElmer, Wellesley, MA).

### **Immunoprecipitation**

For *in vivo* SUMOylation assay, COS-1 cells were transfected with indicated plasmids. At 24 hr after transfection, cells were lysed with immunoprecipitation buffer (10 mM phosphate buffer, 10 mM Tris, 150 mM NaCl, 1% Triton X-100, and 20 mM NEM [pH 7.5]) containing a protease inhibitor cocktail, and immunoprecipitated with the indicated antibodies or EZview Red Affinity Gel matrix (Sigma, St. Louis, MO). For *in vivo* acetylation assay, HEK-293 cells were transfected with indicated plasmids. After 48-hour transfection, cells were lysed in RIPA buffer containing a protease inhibitor cocktail, 10 mM nicotinamide and

500nM trichostatin A, and immunoprecipitated with the indicated antibodies. For denaturing immunoprecipitation, the cells were lysed in 0.2 ml denaturing IP buffer (1% SDS, 50 mM Tris, 10 mM DTT, [pH 7.5]) and boiled for 5 minutes. Subsequently, denatured proteins were diluted in immunoprecipitation buffer and immunoprecipitated with the indicated antibodies. Immunoprecipitated proteins were resolved by SDS/PAGE and analyzed by immunoblotting.

### **Statistical analysis.**

Data are presented as mean  $\pm$  one standard deviation (SD); p values were calculated using an unpaired two-tailed Student's t test in the Microsoft Excel software.  $P > 0.05$  was considered to be not significant; \*,  $P < 0.05$  and \*\*,  $P < 0.01$  means significant and very significant, respectively.

## References

1. Meluh, P. B., and D. Koshland. 1995. Evidence that the MIF2 gene of *Saccharomyces cerevisiae* encodes a centromere protein with homology to the mammalian centromere protein CENP-C. *Mol Biol Cell* 6:793-807.
2. Okura, T., L. Gong, T. Kamitani, T. Wada, I. Okura, C. F. Wei, H. M. Chang, and E. T. Yeh. 1996. Protection against Fas/APO-1- and tumor necrosis factor-mediated cell death by a novel protein, sentrin. *J Immunol* 157:4277-4281.
3. Boddy, M. N., K. Howe, L. D. Etkin, E. Solomon, and P. S. Freemont. 1996. PIC 1, a novel ubiquitin-like protein which interacts with the PML component of a multiprotein complex that is disrupted in acute promyelocytic leukaemia. *Oncogene* 13:971-982.
4. Matunis, M. J., E. Coutavas, and G. Blobel. 1996. A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol* 135:1457-1470.
5. Shen, Z., P. E. Pardington-Purtymun, J. C. Comeaux, R. K. Moyzis, and D. J. Chen. 1996. UBL1, a human ubiquitin-like protein associating with human RAD51/RAD52 proteins. *Genomics* 36:271-279.
6. Mahajan, R., C. Delphin, T. Guan, L. Gerace, and F. Melchior. 1997. A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* 88:97-107.

7. Mannen, H., H. M. Tseng, C. L. Cho, and S. S. Li. 1996. Cloning and expression of human homolog HSMT3 to yeast SMT3 suppressor of MIF2 mutations in a centromere protein gene. *Biochem Biophys Res Commun* 222:178-180.
8. Bayer, P., A. Arndt, S. Metzger, R. Mahajan, F. Melchior, R. Jaenicke, and J. Becker. 1998. Structure determination of the small ubiquitin-related modifier SUMO-1. *J Mol Biol* 280:275-286.
9. Tatham, M. H., E. Jaffray, O. A. Vaughan, J. M. Desterro, C. H. Botting, J. H. Naismith, and R. T. Hay. 2001. Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J Biol Chem* 276:35368-35374.
10. Bohren, K. M., V. Nadkarni, J. H. Song, K. H. Gabbay, and D. Owerbach. 2004. A M55V polymorphism in a novel SUMO gene (SUMO-4) differentially activates heat shock transcription factors and is associated with susceptibility to type I diabetes mellitus. *J Biol Chem* 279:27233-27238.
11. Guo, D., M. Li, Y. Zhang, P. Yang, S. Eckenrode, D. Hopkins, W. Zheng, S. Purohit, R. H. Podolsky, A. Muir, J. Wang, Z. Dong, T. Brusko, M. Atkinson, P. Pozzilli, A. Zeidler, L. J. Raffel, C. O. Jacob, Y. Park, M. Serrano-Rios, M. T. Larrad, Z. Zhang, H. J. Garchon, J. F. Bach, J. I. Rotter, J. X. She, and C. Y. Wang. 2004. A functional variant of SUMO4, a new I kappa B alpha modifier, is associated with type 1 diabetes. *Nat Genet* 36:837-841.
12. Geiss-Friedlander, R., and F. Melchior. 2007. Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol* 8:947-956.

13. Hay, R. T. 2005. SUMO: a history of modification. *Mol Cell* 18:1-12.
14. Li, S. J., and M. Hochstrasser. 1999. A new protease required for cell-cycle progression in yeast. *Nature* 398:246-251.
15. Li, S. J., and M. Hochstrasser. 2000. The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein. *Mol Cell Biol* 20:2367-2377.
16. Gong, L., S. Millas, G. G. Maul, and E. T. Yeh. 2000. Differential regulation of sentrinized proteins by a novel sentrin-specific protease. *J Biol Chem* 275:3355-3359.
17. Hay, R. T. 2007. SUMO-specific proteases: a twist in the tail. *Trends Cell Biol* 17:370-376.
18. Mukhopadhyay, D., and M. Dasso. 2007. Modification in reverse: the SUMO proteases. *Trends Biochem Sci* 32:286-295.
19. Yeh, E. T. 2009. SUMOylation and De-SUMOylation: wrestling with life's processes. *J Biol Chem* 284:8223-8227.
20. Yamaguchi, T., P. Sharma, M. Athanasiou, A. Kumar, S. Yamada, and M. R. Kuehn. 2005. Mutation of SENP1/SuPr-2 reveals an essential role for desumoylation in mouse development. *Mol Cell Biol* 25:5171-5182.
21. Cheng, J., X. Kang, S. Zhang, and E. T. Yeh. 2007. SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. *Cell* 131:584-595.

22. Yu, L., W. Ji, H. Zhang, M. J. Renda, Y. He, S. Lin, E. C. Cheng, H. Chen, D. S. Krause, and W. Min. 2010. SENP1-mediated GATA1 deSUMOylation is critical for definitive erythropoiesis. *J Exp Med* 207:1183-1195.
23. Chiu, S. Y., N. Asai, F. Costantini, and W. Hsu. 2008. SUMO-specific protease 2 is essential for modulating p53-Mdm2 in development of trophoblast stem cell niches and lineages. *PLoS Biol* 6:e310.
24. Kang, X., Y. Qi, Y. Zuo, Q. Wang, Y. Zou, R. J. Schwartz, J. Cheng, and E. T. Yeh. 2010. SUMO-specific protease 2 is essential for suppression of polycomb group protein-mediated gene silencing during embryonic development. *Mol Cell* 38:191-201.
25. Dou, H., C. Huang, M. Singh, P. B. Carpenter, and E. T. Yeh. 2010. Regulation of DNA repair through deSUMOylation and SUMOylation of replication protein A complex. *Mol Cell* 39:333-345.
26. Yang, X. J., and S. Gregoire. 2006. A recurrent phospho-sumoyl switch in transcriptional repression and beyond. *Mol Cell* 23:779-786.
27. Hietakangas, V., J. K. Ahlskog, A. M. Jakobsson, M. Hellesuo, N. M. Sahlberg, C. I. Holmberg, A. Mikhailov, J. J. Palvimo, L. Pirkkala, and L. Sistonen. 2003. Phosphorylation of serine 303 is a prerequisite for the stress-inducible SUMO modification of heat shock factor 1. *Mol Cell Biol* 23:2953-2968.
28. Hietakangas, V., J. Anckar, H. A. Blomster, M. Fujimoto, J. J. Palvimo, A. Nakai, and L. Sistonen. 2006. PDSM, a motif for phosphorylation-dependent SUMO modification. *Proc Natl Acad Sci U S A* 103:45-50.

29. Desterro, J. M., M. S. Rodriguez, and R. T. Hay. 1998. SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. *Mol Cell* 2:233-239.
30. Sapetschnig, A., G. Rischitor, H. Braun, A. Doll, M. Schergaut, F. Melchior, and G. Suske. 2002. Transcription factor Sp3 is silenced through SUMO modification by PIAS1. *EMBO J* 21:5206-5215.
31. Shalizi, A., B. Gaudilliere, Z. Yuan, J. Stegmuller, T. Shirogane, Q. Ge, Y. Tan, B. Schulman, J. W. Harper, and A. Bonni. 2006. A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation. *Science* 311:1012-1017.
32. Gill, G. 2004. SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev* 18:2046-2059.
33. Gill, G. 2005. Something about SUMO inhibits transcription. *Curr Opin Genet Dev* 15:536-541.
34. Perry, J. J., J. A. Tainer, and M. N. Boddy. 2008. A simultaneous role for SUMO and ubiquitin. *Trends Biochem Sci* 33:201-208.
35. Huang, W., S. Ghisletti, K. Saijo, M. Gandhi, M. Aouadi, G. J. Tesz, D. X. Zhang, J. Yao, M. P. Czech, B. L. Goode, M. G. Rosenfeld, and C. K. Glass. 2011. Coronin 2A mediates actin-dependent de-repression of inflammatory response genes. *Nature* 470:414-418.
36. Xie, Y., E. M. Rubenstein, T. Matt, and M. Hochstrasser. 2010. SUMO-independent in vivo activity of a SUMO-targeted ubiquitin ligase toward a short-lived transcription factor. *Genes Dev* 24:893-903.



37. Prudden, J., S. Pebernard, G. Raffa, D. A. Slavin, J. J. Perry, J. A. Tainer, C. H. McGowan, and M. N. Boddy. 2007. SUMO-targeted ubiquitin ligases in genome stability. *EMBO J* 26:4089-4101.
38. Shuai, K., and B. Liu. 2005. Regulation of gene-activation pathways by PIAS proteins in the immune system. *Nat Rev Immunol* 5:593-605.
39. Fu, X. Y., C. Schindler, T. Improt, R. Aebersold, and J. E. Darnell, Jr. 1992. The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction. *Proc Natl Acad Sci U S A* 89:7840-7843.
40. Schindler, C., X. Y. Fu, T. Improt, R. Aebersold, and J. E. Darnell, Jr. 1992. Proteins of transcription factor ISGF-3: one gene encodes the 91-and 84-kDa ISGF-3 proteins that are activated by interferon alpha. *Proc Natl Acad Sci U S A* 89:7836-7839.
41. Levy, D. E., and J. E. Darnell, Jr. 2002. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 3:651-662.
42. Copeland, N. G., D. J. Gilbert, C. Schindler, Z. Zhong, Z. Wen, J. E. Darnell, Jr., A. L. Mui, A. Miyajima, F. W. Quelle, J. N. Ihle, and et al. 1995. Distribution of the mammalian Stat gene family in mouse chromosomes. *Genomics* 29:225-228.
43. Jenuwein, T., and C. D. Allis. 2001. Translating the histone code. *Science* 293:1074-1080.
44. Fischle, W., Y. Wang, and C. D. Allis. 2003. Binary switches and modification cassettes in histone biology and beyond. *Nature* 425:475-479.

45. Lachner, M., R. J. O'Sullivan, and T. Jenuwein. 2003. An epigenetic road map for histone lysine methylation. *J Cell Sci* 116:2117-2124.
46. Vaquero, A., A. Loyola, and D. Reinberg. 2003. The constantly changing face of chromatin. *Sci Aging Knowledge Environ* 2003:RE4.
47. Yang, X. J., and E. Seto. 2008. Lysine acetylation: codified crosstalk with other posttranslational modifications. *Mol Cell* 31:449-461.
48. Paulson, M., S. Pisharody, L. Pan, S. Guadagno, A. L. Mui, and D. E. Levy. 1999. Stat protein transactivation domains recruit p300/CBP through widely divergent sequences. *J Biol Chem* 274:25343-25349.
49. Korzus, E., J. Torchia, D. W. Rose, L. Xu, R. Kurokawa, E. M. McInerney, T. M. Mullen, C. K. Glass, and M. G. Rosenfeld. 1998. Transcription factor-specific requirements for coactivators and their acetyltransferase functions. *Science* 279:703-707.
50. Zhang, J. J., U. Vinkemeier, W. Gu, D. Chakravarti, C. M. Horvath, and J. E. Darnell, Jr. 1996. Two contact regions between Stat1 and CBP/p300 in interferon gamma signaling. *Proc Natl Acad Sci U S A* 93:15092-15096.
51. Bhattacharya, S., R. Eckner, S. Grossman, E. Oldread, Z. Arany, A. D'Andrea, and D. M. Livingston. 1996. Cooperation of Stat2 and p300/CBP in signalling induced by interferon-alpha. *Nature* 383:344-347.
52. Shankaranarayanan, P., P. Chaitidis, H. Kuhn, and S. Nigam. 2001. Acetylation by histone acetyltransferase CREB-binding protein/p300 of STAT6 is required for transcriptional activation of the 15-lipoxygenase-1 gene. *J Biol Chem* 276:42753-42760.

53. Kramer, O. H., S. K. Knauer, G. Greiner, E. Jandt, S. Reichardt, K. H. Guhrs, R. H. Stauber, F. D. Bohmer, and T. Heinzel. 2009. A phosphorylation-acetylation switch regulates STAT1 signaling. *Genes Dev* 23:223-235.
54. Kramer, O. H., D. Baus, S. K. Knauer, S. Stein, E. Jager, R. H. Stauber, M. Grez, E. Pfitzner, and T. Heinzel. 2006. Acetylation of Stat1 modulates NF-kappaB activity. *Genes Dev* 20:473-485.
55. Yuan, Z. L., Y. J. Guan, D. Chatterjee, and Y. E. Chin. 2005. Stat3 dimerization regulated by reversible acetylation of a single lysine residue. *Science* 307:269-273.
56. Tang, X., J. S. Gao, Y. J. Guan, K. E. McLane, Z. L. Yuan, B. Ramratnam, and Y. E. Chin. 2007. Acetylation-dependent signal transduction for type I interferon receptor. *Cell* 131:93-105.
57. Ma, L., J. S. Gao, Y. Guan, X. Shi, H. Zhang, M. K. Ayrapetov, Z. Zhang, L. Xu, Y. M. Hyun, M. Kim, S. Zhuang, and Y. E. Chin. 2010. Acetylation modulates prolactin receptor dimerization. *Proc Natl Acad Sci U S A* 107:19314-19319.
58. Chung, C. D., J. Liao, B. Liu, X. Rao, P. Jay, P. Berta, and K. Shuai. 1997. Specific inhibition of Stat3 signal transduction by PIAS3. *Science* 278:1803-1805.
59. Liu, B., J. Liao, X. Rao, S. A. Kushner, C. D. Chung, D. D. Chang, and K. Shuai. 1998. Inhibition of Stat1-mediated gene activation by PIAS1. *Proc Natl Acad Sci U S A* 95:10626-10631.

60. Liu, B., S. Mink, K. A. Wong, N. Stein, C. Getman, P. W. Dempsey, H. Wu, and K. Shuai. 2004. PIAS1 selectively inhibits interferon-inducible genes and is important in innate immunity. *Nat Immunol* 5:891-898.
61. Liu, B., S. Tahk, K. M. Yee, G. Fan, and K. Shuai. 2010. The ligase PIAS1 restricts natural regulatory T cell differentiation by epigenetic repression. *Science* 330:521-525.
62. Long, J., G. Wang, I. Matsuura, D. He, and F. Liu. 2004. Activation of Smad transcriptional activity by protein inhibitor of activated STAT3 (PIAS3). *Proc Natl Acad Sci U S A* 101:99-104.
63. Schmidt, D., and S. Muller. 2003. PIAS/SUMO: new partners in transcriptional regulation. *Cell Mol Life Sci* 60:2561-2574.
64. Ungureanu, D., S. Vanhatupa, N. Kotaja, J. Yang, S. Aittomaki, O. A. Janne, J. J. Palvimo, and O. Silvennoinen. 2003. PIAS proteins promote SUMO-1 conjugation to STAT1. *Blood* 102:3311-3313.
65. Akira, S. 1999. Functional roles of STAT family proteins: lessons from knockout mice. *Stem Cells* 17:138-146.
66. Imada, K., and W. J. Leonard. 2000. The Jak-STAT pathway. *Mol Immunol* 37:1-11.
67. O'Shea, J. J., M. Gadina, and R. D. Schreiber. 2002. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell* 109 Suppl:S121-131.
68. O'Shea, J. J., R. Lahesmaa, G. Vahedi, A. Laurence, and Y. Kanno. 2011. Genomic views of STAT function in CD4(+) T helper cell differentiation. *Nat Rev Immunol* 11:239-250.

69. Durbin, J. E., R. Hackenmiller, M. C. Simon, and D. E. Levy. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* 84:443-450.
70. Meraz, M. A., J. M. White, K. C. Sheehan, E. A. Bach, S. J. Rodig, A. S. Dighe, D. H. Kaplan, J. K. Riley, A. C. Greenlund, D. Campbell, K. Carver-Moore, R. N. DuBois, R. Clark, M. Aguet, and R. D. Schreiber. 1996. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* 84:431-442.
71. Park, C., S. Li, E. Cha, and C. Schindler. 2000. Immune response in Stat2 knockout mice. *Immunity* 13:795-804.
72. Takeda, K., K. Noguchi, W. Shi, T. Tanaka, M. Matsumoto, N. Yoshida, T. Kishimoto, and S. Akira. 1997. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci U S A* 94:3801-3804.
73. Takeda, K., B. E. Clausen, T. Kaisho, T. Tsujimura, N. Terada, I. Forster, and S. Akira. 1999. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* 10:39-49.
74. Laouar, Y., T. Welte, X. Y. Fu, and R. A. Flavell. 2003. STAT3 is required for Flt3L-dependent dendritic cell differentiation. *Immunity* 19:903-912.
75. Panopoulos, A. D., L. Zhang, J. W. Snow, D. M. Jones, A. M. Smith, K. C. El Kasmi, F. Liu, M. A. Goldsmith, D. C. Link, P. J. Murray, and S. S. Watowich. 2006. STAT3 governs distinct pathways in emergency granulopoiesis and mature neutrophils. *Blood* 108:3682-3690.

76. Chou, W. C., D. E. Levy, and C. K. Lee. 2006. STAT3 positively regulates an early step in B-cell development. *Blood* 108:3005-3011.
77. Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7:145-173.
78. Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133:775-787.
79. Littman, D. R., and A. Y. Rudensky. 2010. Th17 and regulatory T cells in mediating and restraining inflammation. *Cell* 140:845-858.
80. O'Shea, J. J., and W. E. Paul. 2010. Mechanisms underlying lineage commitment and plasticity of helper CD4<sup>+</sup> T cells. *Science* 327:1098-1102.
81. Dong, C. 2006. Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells. *Nat Rev Immunol* 6:329-333.
82. Weaver, C. T., R. D. Hatton, P. R. Mangan, and L. E. Harrington. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 25:821-852.
83. Yang, X. O., A. D. Panopoulos, R. Nurieva, S. H. Chang, D. Wang, S. S. Watowich, and C. Dong. 2007. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem* 282:9358-9363.
84. Burchill, M. A., J. Yang, C. Vogtenhuber, B. R. Blazar, and M. A. Farrar. 2007. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3<sup>+</sup> regulatory T cells. *J Immunol* 178:280-290.

85. Thierfelder, W. E., J. M. van Deursen, K. Yamamoto, R. A. Tripp, S. R. Sarawar, R. T. Carson, M. Y. Sangster, D. A. Vignali, P. C. Doherty, G. C. Grosveld, and J. N. Ihle. 1996. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 382:171-174.
86. Kaplan, M. H., Y. L. Sun, T. Hoey, and M. J. Grusby. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382:174-177.
87. Shimoda, K., J. van Deursen, M. Y. Sangster, S. R. Sarawar, R. T. Carson, R. A. Tripp, C. Chu, F. W. Quelle, T. Nosaka, D. A. Vignali, P. C. Doherty, G. Grosveld, W. E. Paul, and J. N. Ihle. 1996. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature* 380:630-633.
88. Takeda, K., T. Tanaka, W. Shi, M. Matsumoto, M. Minami, S. Kashiwamura, K. Nakanishi, N. Yoshida, T. Kishimoto, and S. Akira. 1996. Essential role of Stat6 in IL-4 signalling. *Nature* 380:627-630.
89. Wakao, H., F. Gouilleux, and B. Groner. 1994. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. *EMBO J* 13:2182-2191.
90. Hennighausen, L., and G. W. Robinson. 2008. Interpretation of cytokine signaling through the transcription factors STAT5A and STAT5B. *Genes Dev* 22:711-721.

91. Kondo, M., I. L. Weissman, and K. Akashi. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 91:661-672.
92. Cobaleda, C., and M. Busslinger. 2008. Developmental plasticity of lymphocytes. *Curr Opin Immunol* 20:139-148.
93. Adolfsson, J., R. Mansson, N. Buza-Vidas, A. Hultquist, K. Liuba, C. T. Jensen, D. Bryder, L. Yang, O. J. Borge, L. A. Thoren, K. Anderson, E. Sitnicka, Y. Sasaki, M. Sigvardsson, and S. E. Jacobsen. 2005. Identification of Flt3<sup>+</sup> lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell* 121:295-306.
94. Welner, R. S., R. Pelayo, and P. W. Kincade. 2008. Evolving views on the genealogy of B cells. *Nat Rev Immunol* 8:95-106.
95. Godfrey, D. I., J. Kennedy, T. Suda, and A. Zlotnik. 1993. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J Immunol* 150:4244-4252.
96. Hardy, R. R., and K. Hayakawa. 2001. B cell development pathways. *Annu Rev Immunol* 19:595-621.
97. Busslinger, M. 2004. Transcriptional control of early B cell development. *Annu Rev Immunol* 22:55-79.
98. Mazzucchelli, R., and S. K. Durum. 2007. Interleukin-7 receptor expression: intelligent design. *Nat Rev Immunol* 7:144-154.



99. von Freeden-Jeffry, U., P. Vieira, L. A. Lucian, T. McNeil, S. E. Burdach, and R. Murray. 1995. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med* 181:1519-1526.
100. Goodwin, R. G., D. Friend, S. F. Ziegler, R. Jerzy, B. A. Falk, S. Gimpel, D. Cosman, S. K. Dower, C. J. March, A. E. Namen, and et al. 1990. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. *Cell* 60:941-951.
101. Noguchi, M., Y. Nakamura, S. M. Russell, S. F. Ziegler, M. Tsang, X. Cao, and W. J. Leonard. 1993. Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. *Science* 262:1877-1880.
102. Malin, S., S. McManus, and M. Busslinger. 2010. STAT5 in B cell development and leukemia. *Curr Opin Immunol* 22:168-176.
103. Leonard, W. J., and J. J. O'Shea. 1998. Jaks and STATs: biological implications. *Annu Rev Immunol* 16:293-322.
104. Schmitt, T. M., and J. C. Zuniga-Pflucker. 2002. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* 17:749-756.
105. Vieira, P., and A. Cumano. 2004. Differentiation of B lymphocytes from hematopoietic stem cells. *Methods Mol Biol* 271:67-76.
106. Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68:869-877.

107. Hoelbl, A., B. Kovacic, M. A. Kerenyi, O. Simma, W. Warsch, Y. Cui, H. Beug, L. Hennighausen, R. Moriggl, and V. Sexl. 2006. Clarifying the role of Stat5 in lymphoid development and Abelson-induced transformation. *Blood* 107:4898-4906.
108. Yao, Z., Y. Cui, W. T. Watford, J. H. Bream, K. Yamaoka, B. D. Hissong, D. Li, S. K. Durum, Q. Jiang, A. Bhandoola, L. Hennighausen, and J. J. O'Shea. 2006. Stat5a/b are essential for normal lymphoid development and differentiation. *Proc Natl Acad Sci U S A* 103:1000-1005.
109. Malin, S., S. McManus, C. Cobaleda, M. Novatchkova, A. Delogu, P. Bouillet, A. Strasser, and M. Busslinger. 2010. Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B cell development. *Nat Immunol* 11:171-179.
110. Rogers, R. S., C. M. Horvath, and M. J. Matunis. 2003. SUMO modification of STAT1 and its role in PIAS-mediated inhibition of gene activation. *J Biol Chem* 278:30091-30097.
111. Song, L., S. Bhattacharya, A. A. Yunus, C. D. Lima, and C. Schindler. 2006. Stat1 and SUMO modification. *Blood* 108:3237-3244.
112. Pfitzner, E., R. Jahne, M. Wissler, E. Stoecklin, and B. Groner. 1998. p300/CREB-binding protein enhances the prolactin-mediated transcriptional induction through direct interaction with the transactivation domain of Stat5, but does not participate in the Stat5-mediated suppression of the glucocorticoid response. *Mol Endocrinol* 12:1582-1593.

113. Cao, X., E. W. Shores, J. Hu-Li, M. R. Anver, B. L. Kelsall, S. M. Russell, J. Drago, M. Noguchi, A. Grinberg, E. T. Bloom, and et al. 1995. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. *Immunity* 2:223-238.
114. DiSanto, J. P., W. Muller, D. Guy-Grand, A. Fischer, and K. Rajewsky. 1995. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. *Proc Natl Acad Sci U S A* 92:377-381.
115. Nosaka, T., J. M. van Deursen, R. A. Tripp, W. E. Thierfelder, B. A. Witthuhn, A. P. McMickle, P. C. Doherty, G. C. Grosveld, and J. N. Ihle. 1995. Defective lymphoid development in mice lacking Jak3. *Science* 270:800-802.
116. Peschon, J. J., P. J. Morrissey, K. H. Grabstein, F. J. Ramsdell, E. Maraskovsky, B. C. Gliniak, L. S. Park, S. F. Ziegler, D. E. Williams, C. B. Ware, J. D. Meyer, and B. L. Davison. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* 180:1955-1960.
117. Thomis, D. C., C. B. Gurniak, E. Tivol, A. H. Sharpe, and L. J. Berg. 1995. Defects in B lymphocyte maturation and T lymphocyte activation in mice lacking Jak3. *Science* 270:794-797.
118. Noguchi, M., H. Yi, H. M. Rosenblatt, A. H. Filipovich, S. Adelstein, W. S. Modi, O. W. McBride, and W. J. Leonard. 1993. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell* 73:147-157.

119. Macchi, P., A. Villa, S. Giliani, M. G. Sacco, A. Frattini, F. Porta, A. G. Ugazio, J. A. Johnston, F. Candotti, J. J. O'Shea, and et al. 1995. Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). *Nature* 377:65-68.
120. Russell, S. M., N. Tayebi, H. Nakajima, M. C. Riedy, J. L. Roberts, M. J. Aman, T. S. Migone, M. Noguchi, M. L. Markert, R. H. Buckley, J. J. O'Shea, and W. J. Leonard. 1995. Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. *Science* 270:797-800.
121. Puel, A., S. F. Ziegler, R. H. Buckley, and W. J. Leonard. 1998. Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. *Nat Genet* 20:394-397.
122. Maraskovsky, E., L. A. O'Reilly, M. Teepe, L. M. Corcoran, J. J. Peschon, and A. Strasser. 1997. Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1<sup>-/-</sup> mice. *Cell* 89:1011-1019.
123. Akashi, K., M. Kondo, U. von Freeden-Jeffry, R. Murray, and I. L. Weissman. 1997. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. *Cell* 89:1033-1041.
124. Kondo, M., K. Akashi, J. Domen, K. Sugamura, and I. L. Weissman. 1997. Bcl-2 rescues T lymphopoiesis, but not B or NK cell development, in common gamma chain-deficient mice. *Immunity* 7:155-162.

125. Kikuchi, K., A. Y. Lai, C. L. Hsu, and M. Kondo. 2005. IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF. *J Exp Med* 201:1197-1203.
126. Teglund, S., C. McKay, E. Schuetz, J. M. van Deursen, D. Stravopodis, D. Wang, M. Brown, S. Bodner, G. Grosveld, and J. N. Ihle. 1998. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell* 93:841-850.
127. Moriggl, R., V. Sexl, R. Piekorz, D. Topham, and J. N. Ihle. 1999. Stat5 activation is uniquely associated with cytokine signaling in peripheral T cells. *Immunity* 11:225-230.
128. Sexl, V., R. Piekorz, R. Moriggl, J. Rohrer, M. P. Brown, K. D. Bunting, K. Rothhammer, M. F. Roussel, and J. N. Ihle. 2000. Stat5a/b contribute to interleukin 7-induced B-cell precursor expansion, but abl- and bcr/abl-induced transformation are independent of stat5. *Blood* 96:2277-2283.
129. Schmidt, D., and S. Muller. 2002. Members of the PIAS family act as SUMO ligases for c-Jun and p53 and repress p53 activity. *Proc Natl Acad Sci U S A* 99:2872-2877.
130. Kahyo, T., T. Nishida, and H. Yasuda. 2001. Involvement of PIAS1 in the sumoylation of tumor suppressor p53. *Mol Cell* 8:713-718.
131. Sachdev, S., L. Bruhn, H. Sieber, A. Pichler, F. Melchior, and R. Grosschedl. 2001. PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. *Genes Dev* 15:3088-3103.

132. Strahl, B. D., and C. D. Allis. 2000. The language of covalent histone modifications. *Nature* 403:41-45.
133. Cao, R., L. Wang, H. Wang, L. Xia, H. Erdjument-Bromage, P. Tempst, R. S. Jones, and Y. Zhang. 2002. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 298:1039-1043.
134. Jacobs, S. A., S. D. Taverna, Y. Zhang, S. D. Briggs, J. Li, J. C. Eissenberg, C. D. Allis, and S. Khorasanizadeh. 2001. Specificity of the HP1 chromo domain for the methylated N-terminus of histone H3. *EMBO J* 20:5232-5241.
135. Gregoire, S., A. M. Tremblay, L. Xiao, Q. Yang, K. Ma, J. Nie, Z. Mao, Z. Wu, V. Giguere, and X. J. Yang. 2006. Control of MEF2 transcriptional activity by coordinated phosphorylation and sumoylation. *J Biol Chem* 281:4423-4433.
136. Jakobs, A., J. Koehnke, F. Himstedt, M. Funk, B. Korn, M. Gaestel, and R. Niedenthal. 2007. Ubc9 fusion-directed SUMOylation (UFDS): a method to analyze function of protein SUMOylation. *Nat Methods* 4:245-250.
137. Zimnik, S., M. Gaestel, and R. Niedenthal. 2009. Mutually exclusive STAT1 modifications identified by Ubc9/substrate dimerization-dependent SUMOylation. *Nucleic Acids Res* 37:e30.
138. Stankovic-Valentin, N., S. Deltour, J. Seeler, S. Pinte, G. Vergoten, C. Guerardel, A. Dejean, and D. Leprince. 2007. An acetylation/deacetylation-SUMOylation switch through a phylogenetically conserved psiKXEP motif in the tumor suppressor HIC1 regulates transcriptional repression activity. *Mol Cell Biol* 27:2661-2675.

139. Cheng, J., N. D. Perkins, and E. T. Yeh. 2005. Differential regulation of c-Jun-dependent transcription by SUMO-specific proteases. *J Biol Chem* 280:14492-14498.
140. Dai, X., Y. Chen, L. Di, A. Podd, G. Li, K. D. Bunting, L. Hennighausen, R. Wen, and D. Wang. 2007. Stat5 is essential for early B cell development but not for B cell maturation and function. *J Immunol* 179:1068-1079.

## VITA

Thang Van Nguyen was born in Hung Yen province, Vietnam on September 02, 1973. His interest in biology has developed since he was a child often assisting his parents in farming and observing domestic animals affected by pathogens in countryside. He decided to pursue Doctor of Veterinary Medicine (D.V.M.) at Hanoi Agriculture University from 1992 to 1997. After completing D.V.M., he worked as a lecturer at Hanoi Agriculture University, where he gave lectures and guided students in the field of Veterinary Medicine. In 2000, the golden chance came to him when the Vietnamese Government opened a big project on “the National Scholarship to the most promising young scientists for studying in developed countries”. Despite of the fact that the project was highly competitive and selective, he was awarded this scholarship. Subsequently, He joined Master Program in Molecular Biology at ‘Interuniversity Program in Molecular Biology’ (IPMB), held by Free Brussels University, Catholic Leuven University and Antwerp University, Belgium from 2001 to 2003. At IPMB, his passion for molecular biology played a vital role in his success of the program. He was totally absorbed by the lectures on various subjects such as biochemistry, virology, microbiology, immunology, and in particular molecular biology presented by the esteemed Professors. In 2004, he got a scholarship from Vietnam Education Foundation (VEF) to pursue a Ph.D. in the US. He entered the Graduate School of Biomedical Sciences at the University of Texas Health Science Center and MD Anderson Cancer Center.