國立臺灣大學醫學院免疫學研究所

博士論文

Department of Graduate Institute of Immunology
College of Medicine
National Taiwan University

Doctoral Dissertation

SUMO化修飾減弱c-Maf相關之介白素四與介白素二十一之表現

SUMOylation attenuates c-Maf-dependent IL-4 and IL-21 expression

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中華民國 九十九 年 七 月

July, 2010

國立臺灣大學博士學位論文
口試委員會審定書

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本論文係林伯修君(學號 D91449003)在國立臺灣大學醫學院免疫學研究所完成之博士學位論文，於民國 99 年 07 月 21 日承下列考試委員審查通過及口試及格，特此證明

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（指導教授）

系主任、所長 許秉寧

Acknowledgement

這段日子承蒙許多人的幫助，該感謝的人太多太多了。首先最要感謝的是我的指導老師繆希椿老師：謝謝老師收了我做弟子，並一步一步的指導我。想起一開始實驗室只有老師和我兩個人，再到如今的人丁興旺。老師和我一起見證了實驗室的萌芽與茁壯，也一起分享研究的樂趣與挑戰。謝謝老師讓我有多方面學習的機會，並耐心的讓我慢慢成長。再來要感謝我的口試委員們：賴明宗老師、司徒惠康老師、許秉寧老師與謝世良老師。這些年來適時的提出建議，讓我的研究能順利進行下去。同時也感謝我的免疫學啟蒙老師：江伯倫老師。沒有江老師的啟發，我也不會走上研究免疫的這條路。此外也要感謝所上的老師們：伍安怡老師、李建國老師與顧家□老師。謝謝老師們在研究與生活上的鼓勵，讓我有勇氣在面對挫折時能堅持下去。

再來要謝謝這些年來在實驗室一起拼命的夥伴們：博士班的正彥、芷君、孝瑋、彰憲；碩士班的婉云、俊國、偉峰、佩芸、雅各、夢薇、偉豪、安翔、馥瑋、益立、宜緯、茹愷與欣穎 (真的是歷屆成員的回顧史呀！)。大家能一起共事真是難得的緣分，也感謝大家在實驗還有生活上的大力幫忙。此外還有許多許多免疫所的其他夥伴們，謝謝大家在這八年中的鼓勵與幫助，這些都將是我美好的回憶。

最後要謝謝我的家人，給我一個溫暖的避風港，讓我能無後顧之憂的做學問。最感謝親愛的老婆：珮愉，這八年你辛苦了。我可愛的女兒小倢□，謝謝你選擇來到這個家庭陪伴我們。感謝兩對爸爸媽媽，這些年來讓你們操心了。祝福大家

平安快樂，也將此論文與我的喜悅與大家分享，謝謝大家。

八年的時間在人生中是一段不算短的歲月，這段期間應該是我人生中變化最大的時期了。唸博士班、寫paper、結婚、有小孩、買房子的事情一件件的來，喜怒哀樂通通都有。回想起來雖然辛苦，但是也都是值得珍惜的回憶。這本論文是這八年歲月的里程碑。很多人告訴我：拿到博士學位只是得到人生的入場卷而已。回首過去，展望未來，越過這個分界點，我將繼續努力迎向下一個八年。

中文摘要

轉錄因子的功能是藉由控制其生成、活性與降解來嚴格調控的，而SUMO化修飾是在轉錄後層級調控蛋白質的活性。c-Maf蛋白是屬於大Maf家族的鹼性白氨酸拉鍊蛋白，在第二或第十七型輔助T細胞上分別為介白素四與介白素二十一的專一性轉錄因子。我們藉由酵母雙雜交系統來尋找c-Maf的交互作用蛋白。我們發現兩個SUMO化修飾的關鍵酵素：Ubc9與PIAS1，可以與c-Maf蛋白交互作用。在T細胞中，PIAS1可以與c-Maf蛋白交互作用；這兩個SUMO化修飾的關鍵酵素也與c-Maf蛋白共同位於細胞核中。我們也確認c-Maf蛋白在活體外與活體內都可被SUMO修飾。c-Maf蛋白N端第33個離胺酸是SUMO蛋白的修飾位。SUMO化修飾會降低c-Maf蛋白對介白素四的轉錄活性，而無法被SUMO化修飾的c-Maf蛋白卻有較強的轉錄活性。同時，我們也發現c-Maf是介白素二十一的專一性轉錄因子。SUMO化修飾也會影響c-Maf蛋白相關之介白素二十一的產生。SUMO化修飾不會影響c-Maf蛋白的穩定性，但無法SUMO化修飾的c-Maf蛋白匯集到啟動子的能力卻較強。我們的結論是c-Maf蛋白的SUMO化修飾對其在輔助T細胞中的活性十分重要。

Abstract

The function of transcription factor is tightly regulated by controlling their synthesis, activity and degradation. SUMOylation modulates target protein activity on post-translational level. c-Maf, the cellular homologue of v-Maf, is a basic-leucine zipper protein and belongs to the large Maf family. In helper T cells, c-Maf is the specific transcription factor of the IL-4 and IL-21 genes in type 2 T helper (Th2) and type 17 T helper (Th17) cells, respectively. In our study, we performed the yeast two-hybrid assay to identify the c-Maf interacting proteins. We found that c-Maf can interact with Ubc9 and PIAS1, the key enzymes of SUMOylation system. In T cells, c-Maf interacts with PIAS1 in primary T cells and also co-localizes with these two SUMO ligases in the nucleus. We also demonstrated that c-Maf can be SUMOylated *in vitro* and also *in vivo*. We identified the c-Maf SUMO acceptor site(s) by mutated the putative conjugating lysine residues. We demonstrated that N-terminal lysine-33 within the activation domain is the SUMO acceptor site for c-Maf. SUMO modification attenuates Wt-c-Maf transcriptional activity. Conversely, c-Maf SUMO deficient mutant is more potent to drive IL-4 production in Th2 cells. Furthermore, we showed that c-Maf, but not other transcription factor, transactivates IL-21 gene expression. SUMOylation also affects c-Maf dependent IL-21 production. In addition, SUMO deficient c-Maf does not alter the localization and the protein stability, but further enhances its recruitment to the *Il4-promoter*. We conclude that post-translational lysine-33 SUMOylation is critical for c-Maf activity in helper T cells.

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Chapter I Introduction

1.1 T helper cell lineages and their differentiation

CD4 T lymphocytes play an important role in adaptive immunity. They help B cells to produce antibodies, modulate the function of macrophage, protect against a variety of pathogens and prevent the autoimmune diseases. Upon encountering antigen, naïve $CD4^+$ precursor helper T (Th) cells differentiate into distinct effector Th cells. For over twenty years, our knowledge of effector helper T cell subsets is limited to Th1/Th2 cells. The "Th1/Th2 Paradigm" was firstly introduced by Mossman and Coffman (Mosmann et al., 1986), and subsequently by Bottomly's group (Killar et al., 1987). During the past few years, several distinct helper T subsets were identified and characterized by the immunologists. Two major new types of effector Th lineages are the Th17 (Harrington et al., 2005; Park et al., 2005) and the regulatory T (Treg) (Chen et al., 2005; Fu et al., 2004). Another two types of Th cells, the follicular helper T (Tfh) (King et al., 2008) and the Th9 (Veldhoen et al., 2008), are also been characterized and studied. Until now, the family members of Th have grown into six lineages.

The fates of Th cells were determined by the microenvironment of their surrounding. Different combinations of cytokine are required for distinct helper T cell differentiation. Th1 cells need IL-12 and IFN-γ, Th2 cells need IL-4, Th17 need TGF-β, IL-6, IL-23 and IL-17, and iTreg cells need TGF-β and IL-2 for their lineage development. The classification of effector Th cell subsets is also categorized by their cytokine secretion patterns and their effector functions. Th1 cells produce interferon-gamma (IFN-γ), lymphotoxin (LT) and lots of interleukin-2 (IL-2). Th1 responses have been described to

be important in defense of intracellular pathogens and responsible for mounting organ-specific autoreactive immune responses. Conversely, Th2 cells produce IL-4, IL-5 and IL-13. Th2 responses are critical in anti-helminthic and anti-inflammatory reactions. Th17 cells produce IL-17A, IL-17F, IL-22 and IL-21 as well. Th17 cells play key roles during inflammation and tissue injury. They are also important to protect against the microbial (for example, *Candida* and *Staphylococcus*) at the mucosal. Loss of Th17 cells may lead to the opportunistic infections. Furthermore, the autoimmune diseases such as multiple sclerosis, psoriasis, autoimmune uveitis, juvenile diabetes and rheumatoid are also Th17-related. iTreg cells produce TGF-β and IL-10. Their function is to suppress and to maintain the homeostasis of immune responses, just like the nature Treg cells (nTreg) do.

Determination of the Th lineage is controlled by the expression of specific transcription factors. Each Th lineages have their unique master transcription factors, T-bet, GATA3, RORγt, and Foxp3 are the major specific transcription factor for the development of Th1, Th2, Th17, and Treg effector cells, respectively. On Th1 cells, T-bet is a Th1-specific transcription factor of IFN-γ (Szabo et al., 2000a). T-bet deficient mice exhibit Th2 phenotype and fail to mount Th1 immune responses (Szabo et al., 2002a). IFN-γ is critical for Th1 cell differentiation. It can activate the signal transducer and activator of transcription 1 (STAT1). STAT1 will induce the expression of T-bet (Lighvani et al., 2001). T-bet can make a positive feedback loop of IFN-γ by remodeling the IFN-γ allele. Overexpression of T-bet can force the naïve or Th2 cells to develop into Th1-like phenotype (Mullen et al., 2002; Szabo et al., 2000b). T-bet knockout mice have the defect in Th1 immune response both in vitro and in vivo (Szabo et al., 2002b). On Th2 cells, GATA-3, a zinc finger protein of GATA family, is first

cloned as a Th2 specific transcription factor (Ho et al., 1991; Ko et al., 1991). GATA-3 is essential for normal embryonic development as well as for the generation of T cell lineage (Pandolfi et al., 1995; Ting et al., 1996). It plays a role to block Th1 development through downregulation of STAT4 (Usui et al., 2003). GATA3 does not seem to regulate IL-4 transcription directly (Ouyang et al., 1998). However, It may instead act as a chromatin-remodeling factor, to make the IL-4/IL-5 locus accessible, thus effecting a global control of Th2 cytokine production (Lee et al., 2000; Ouyang et al., 2000). Th17 cells express high level of RORγt, but not GATA3 and T-bet (Ivanov et al., 2006). RORγt deficient cells produce few IL-17, and the RORγt deficient mice are resistant to experimental autoimmune encephalomyelitis (EAE), which is a Th17-mediated disease model (Yang et al., 2008). On Treg cells, Foxp3 is essential for its suppressive activity. Forced expression of Foxp3 by retroviral Foxp3 on conventional T cells acquired a Treg phenotype (Fontenot et al., 2003). In addition, inhibiting the Foxp3 on Treg cells will develop into Th2-like phenotype cells (Wan and Flavell, 2007).

Unlike these master transcription regulators, other transcription factors control only a few of genes and the expression of these factors are not lineage specific. They can work on different cell types and have the different effects. The transcription factor c-Maf is a good example, which specifically controls the IL-4 and IL-21 cytokines expression in Th2 and Th17 cells, respectively (Bauquet et al., 2009; Hiramatsu et al., ; Ho et al., 1996) .

1.2 Overview of c-Maf

c-Maf belongs to the maf family. The first Maf family member, v-maf oncogene was identified in 1989. It was originally discovered as the transduced transforming component of avian **m**usculo**a**poneurotic **f**ibrosarcoma virus, AS42 (Nishizawa et al., 1989). Subsequent studies identify its cellular homologue, and named c-maf (Kataoka et al., 1993). After that, other maf memebers were found subsequently.

Members of the maf family are divided into two subgroups: the large Mafs and the small Mafs. The large maf proteins, including c-maf, maf-B (Kataoka et al., 1994a), NRL and maf-L/A (Ogino and Yasuda, 1998), all contain a distinctive acidic domain that probably enables transcriptional activation. The small maf proteins, including maf-K, maf-F (Fujiwara et al., 1993) and maf-G (Kataoka et al., 1995), all lack N-terminal activation domain (AD). The amino-terminal domain of maf, the activation domain, is conserved among these family members and is rich in acidic and serine, threonine, and proline residues. This region is essential for transcriptional activation. The central part of v-/c-maf, the hinge domain, contains a cluster of histidine residues and three clusters of glycine residues. The histidine cluster is also found in maf-A/L and maf-B, but the glycine clusters are unique to the v-/c-maf protein. A new domain (called minimal transactivation domain, MTD) inside the activation domain was characterized (Friedman et al., 2004). It contain 35 amino acid residues in the proline- and serine-rich N-terminal region, which exhibited activation of target promoters when combined with Lex A or Gal 4 DNA binding domains in the yeast. MTD interacted with full-length or the C-terminal domain of TATA-binding protein (TBP). These interactions were also found in all other large maf family proteins, including c-maf. C-terminal region of maf family proteins shares a common, highly-conserved basic region and leucine zipper

(b-Zip) motif which mediates DNA binding and dimmerization. This domain has the transactivator function and transforming ability (Blank and Andrews, 1997; Kataoka et al., 1993).

The specific DNA binding sequence of maf protein is named MARE (**ma**f **r**ecognition **e**lement). The consensus MARE has been identified (Kataoka et al., 1994b). Maf binds to the MARE site by forming the homodimers or the heterodimers with related b-Zip proteins. The MARE motif is similar to the TRE [phorbol-12-O tetradecanoate-13-acetate (TPA) -responsive element] and CRE (cAMP responsive element). Maf dimers bind to a 13 bp palindromic sequence TPA-MARE (T-MARE) or a 14 bp palindromic sequence CRE-type MARE (C-MARE) (Kataoka et al., 1994a; Kerppola and Curran, 1994). The MARE site on *Il4* gene proximal promoter is an atypical, half, MARE site (Ho et al., 1996).

1.3 The functions of c-Maf in immune system

c-Maf is well known to be a specific transcription factor for *Il4* gene expression. Unlike GATA3, c-maf controls the expression of IL-4. In 1996, c-Maf was reported to be highly expressed in Th2 cells not Th1 lineage, and c-Maf promots IL-4 production by binding to the *Il4* gene proximal promoter (Ho et al., 1996). IL-4 is the signature cytokine of Th2 cells and plays an important role both in humoral and adaptive immune response. IL-4 activates the signal transducer and activate or of transcription 6 (STAT6) through the IL-4 receptor. STAT6 induces the Th2 master regulator GATA-3 expression. However, GATA3 does not directly regulate IL4 gene transcription but acts as a chromatin-remodeling factor to control the global Th2 cytokine production (Lee et al., 2000; Ouyang et al., 2000). IL-4 also secreted by B cell and leads to the isotype

switching to produce IgE, which is important in anti-parasites and allergic responses. Many transcription factors induce IL-4 production by binding to the *Il4* proximal promoter, such as AP-1, NF-κB (p65 and c-REL), NF-AT (NF-ATc1 and NF-ATc2) and C/EBPδ (Rooney, 1995; Li-Webber, 1998; Peng, 2001; Berberich-Siebelt, 2006). Unlike c-Maf, these factors are not the *Il4* gene specific transactivators and exhibit the global effects during T cell differentiation. The c-maf deficient mice showed a severe impairment in the expression of IL-4, but not other Th2 cytokines, both in Th2 cells and in NK1.1 T cells. Interestingly, spleen cells from c-maf deficient mice produced normal levels of IL-13 and IgE, and produced virtually wild-type levels of other Th2 cytokines when differentiating in the presence of exogenous IL-4 (Kim et al., 1999). Independent of IL-4, c-maf is also essential for normal induction of CD25 in developing Th2 cells. The levels of CD25 are significantly higher in developing Th2 cells than Th1 cells during in vitro differentiation.

Recently, c-Maf is found to be highly expressed and play important roles in other T helper cell subsets. Both Th-17 cells and Tfh cells express high level of c-Maf as well as the Th2 subset and that ICOS-deficient mice, that lead to c-Maf short to, results in a defect in IL-21 production and their population expansion (Bauquet et al., 2009). Dr. Kuchroo's study showed that the deficiency of c-Maf results in reduced IL-10 production in Tr1 cells (Pot et al., 2009). They firstly reported that c-Maf transactivate the IL-21 gene directly. IL-21 is a four-helix-bundle type I cytokine. It was produced by Th2 (Wurster et al., 2002), T_{FH} (King et al., 2008), or NKT cells (Harada et al., 2006). IL-21 highly expressed in Th17 cells and promoted the development of Th17 cells (Fantini et al., 2007; Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007). Furthermore, it also promoted the proliferation, differentiation, and functions of T, B,

NK, and dendritic cell's.

In addition to its roles on T cells, c-Maf also has different functions in other immune cells. c-maf is a potent activator of IL-10 gene expression in monocytes and macrophages. Overexpression of c-Maf suppresses IL-12 p40 and p35 gene transcription. Moreover, inhibition of IL-12 p40 gene expression by c-maf requires the N-terminal transactivation domain (Cao et al., 2002). However, IL-4-producing mast cells do not express the c-maf factor. Mutation of a defined c-maf binding site within the proximal IL-4 promoter, which profoundly affects transcription in T cells, has no effect on expression of the *Il4* promoter-luciferase plasmids in mast cells. (Sherman et al., 1999) Therefore, we can conclude that c-Maf regulate different cytokines on different cell types.

1.4 Post-translational modification by SUMOylation: a ubiquitin-like modification system

The activity of transcription factors is controlled not only by their synthesis (both the transcriptional and post-transcriptional level) but also modulated by complicated post-translational modifications. Post-translational modification includes the sulfation, glycosylation, phosphorylation, methylation, lipidation, acetylation, ubiqutination and sumoylation. These modifications may affect their target proteins on bioactivity, localization, dimerization, DNA binding ability, interaction with other partners or stability. SUMOylation has recently been found as an post-transcriptional modifier (Saitoh et al., 1997). SUMO is **s**mall **u**biquitin-like **mo**difier, also known as smt3, sentrin, GMP1 or PIC1 (Melchior, 2000; Muller et al., 2001; Seeler and Dejean, 2003).

SUMO protein is similar to the ubiquitin on three-dimentional structure, although the amino acid sequence is only 18% identity (Bayer et al., 1998). Alternatively, these two proteins are functionally different. Ubiquitination leads the target protein to proteasomal degradation or activation. In contrast, SUMOylation does not appear to target proteins for degradation, and the conjugating of SUMO usually causes the suppression of protein activity. Furthermore, SUMO modification is reversible by SUMO deconjugating isopeptidase, the SUSP/SENP family (Zhao, 2007). Four SUMO isoforms, SUMO-1, SUMO-2, SUMO-3 and SUMO-4, have been identified. Although the mature forms of SUMO-2 and -3 are 95% identical to each other at the amino acid level, they are only 47% identical to SUMO-1 (Saitoh and Hinchey, 2000). SUMO-1 conjugated to proteins as a monomer, while SUMO-2 SUMO-3 and SUMO-4 are conjugated to proteins as polymers (Bohren et al., 2004; Tatham et al., 2001).

Both ubiquitin and SUMO modification use similar enzyme systems to covalently attach a protein modifier on the target protein. Like ubiquitin pathway, SUMO molecules need a serious of enzyme to activate and transport it to their target protein. The SUMO E1 (SAE1/SAE2) activates SUMO and then transfers the activated SUMO to the SUMO E2 ligase (Ubc9). The activated SUMO is further transferred from Ubc9 to target proteins via SUMO E3 ligase, such as RanBP1, PIASs or Pc2. SUMO modification can be reversed by SUMO deconjugating isopeptidase of the SUSP/SENP family, which contain the ubiquitin-like protease (ULP) domain.

Most of the SUMOylation modifications were carried out in the shot motif. This consensus SUMO binding site sequences are **ΨKXE**, where **Ψ** is a large hydrophobic amino acid and **K** (lysine) is the site for SUMO conjugation. The following **X** could be

any peptide and the final **E** means the glutamic acid (Johnson, 2004).

SUMOylation modulates the activity of target transcription factors by recruitment of corepressor (or activator) complexes or mediats the assembling of the repressor (or activator) complexes (Gill, 2004). Although post-translational modification by SUMO has diverse effects on transcription factor activity, in most cases, SUMOylation acts as a suppressor. SUMO conjugation also has other functions, including the stabilizing some proteins, cross-talking with other post-translational modification, disrupting the protein-protein interaction, blocking the transcription factor-DNA binding and altering their subcellular localization (Dohmen, 2004; Muller et al., 2004).

1.5 Rationale and significance:

The regulation network of c-Maf activity is not fully understood. The expression level of c-Maf is up-regulated during Th2 cell differentiation through TCR/CD28 signaling. It has been reported that TCR/CD28 mediated c-Maf expression is Vav1 dependent (Tanaka et al. 2005). IL-6 was also reported to induce c-Maf expression by calcium dependent signaling pathway (Yang, 2005). Recently, ICOS signal was found to induce c-Maf expression on Th-17 and T_{FH} cells (Bauquet, 2009).

In the post-translational level, c-Maf was shown to undergo Serine/threonine phosphorylation by P38 MAP kinase and GSK3 (Sii-Felice, 2005; Rocques, 2007). However, post-translational modification of c-Maf other than phosphorylation is rarely known. By yeast two-hybrid system, we identified two c-Maf interacting proteins, Ubc9 and PIAS1. Two other large Maf proteins, MafB and MafA, have been reported to be

modified by SUMO (Shao and Cobb, 2009; Tillmanns et al., 2007). However, the SUMOylation of c-Maf has not been reported before. In this study, we confirmed that c-maf is modified by SUMO on single conserved lysine residue *in vitro* and *in vivo*. Deficient of SUMO conjugation enhanced the c-Maf activity on IL-4 gene expression. We further demonstrated that the recruitment of c-Maf to the *Il4* promoter is modulated by the SUMOylation.

Chapter II Materials and Methods

Part 1. Materials:

2.1-1 Antibodies

Anti-actin Ab (clone C4)	Chemicon (Millipore), Billerica, MA, USA
LEAF^{TM} Anti-mouse CD3ε Ab (clone 2C11)	Biolegend, San diago, CA, USA
LEAF^{TM} Anti-mouse CD28 Ab (clone 37.51)	Biolegend, San diago, CA, USA
Anti-EGFP Ab (clone ab290)	Abcam, Cambridge, Ma, USA
Anti-Flag Ab (clone M2)	Sigma, St. Louis, MO, USA
Anti-GMP-1 Ab (clone 21C7)	Zymed, San Francisco, CA, USA
Anti-HA Ab (clone 12CA5)	Roche, Mannheim, Germany
Anti-mouse IL-4 Ab (clone 11B11)	eBioscience, San diago, CA, USA
Anti-mouse IFN-γ Ab (clone R4-6A2)	eBioscience, San diago, CA, USA
Anti-c-Maf Ab (clone M-153)	Santa Cruz Biotechnology, Santa cruz, CA, USA
Anti-PIAS1 Ab (clone C-20)	Santa Cruz Biotechnology, Santa cruz, CA, USA
Anti-SUMO-1 Ab (clone D-11)	Santa Cruz Biotechnology, Santa cruz, CA, USA
Anti-Ubc9 Ab (clone N-15)	Santa Cruz Biotechnology, Santa cruz, CA, USA

2.1-2 Cell cultures and mice

HEK293T and RK13 cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 10 mM HEPES, 1% L-glutamine, 1 mM sodium pyruvate, 1X non-essential amino acid, 100 units/ml streptomycin and

penicillin. The Primary CD4 T cells and the DO11.10 cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1 mM sodium pyruvate, 1X non-essential amino acid, 100 units/ml streptomycin and penicillin. These reagents were obtained from Invitrogen (Grand Island, NY). The animal experiments were approved by the Animal Use Committee in National Taiwan University College of Medicine.

2.1-3 Chemicals and reagents

Cycloheximide (CHX)	Sigma, St. Louis, MO, USA
N-Ethylmaleimide (NEM)	Sigma, St. Louis, MO, USA
Ionomycin	Sigma, St. Louis, MO, USA
NaCl	Sigma, St. Louis, MO, USA
NP-40	Sigma, St. Louis, MO, USA
PMSF	Sigma, St. Louis, MO, USA
Phorbol 12-myristate 13-acetate (PMA)	Sigma, St. Louis, MO, USA
Proteinase inhibitor cocktail	Sigma, St. Louis, MO, USA
Tris	USB, Cleveland, Ohio, USA

2.1-4 Cytokines

Recombinate human IL-2	Peprotech, Rocky Hill, NJ, USA
Recombinate mouse IL-4	Peprotech, Rocky Hill, NJ, USA
Recombinate mouse IL-6	Peprotech, Rocky Hill, NJ, USA
Recombinate mouse IL-23	Peprotech, Rocky Hill, NJ, USA
Recombinate human TGF-β	Peprotech, Rocky Hill, NJ, USA

2.1-5 Kits

Dual-Glo™ Luciferase assay system	Promega, Madison, WI, USA
EasySep mouse CD4+ selection kit	Stemcell technology, USA
FastStart Taq DNA polymerase	Roche, Basel, Switzerland
jetPET™ transfection reagent	Polyplus-transfection, New Yark, NY, USA
Quickchange Site-directed mutagenesis kit	Stratagene, La Jolla, CA, USA

2.1-6 Vectors

- **pDsRed-C1-c-Maf:** Wt-c-Maf was cloned into pDsRed-C1 backbone by *EcoRI-SalI* sites.

- **pEF1α/Myc-HisA-Wt- and pEF1α/Myc-HisA-C603S-PIAS1 :** These two vectors were kindly provided by Dr. Hung-wen Chen. Wt- and -C603S-PIAS1 were cloned into EF1α/Myc-HisA (the Myc/His-tag was replaced by 3X Flag-tag) backbone by *KpnI-PmeI* sites.

- **pEGFP-C1-SUMO-1-GG and -AA:** These two vectors were kindly provided by Dr. Hung-wen Chen. SUMO-1-GG and SUMO-1-AA were cloned into pEGFP-C1 backbone by *BglII-HindIII* sites.

- **pEGFP-N1-c-Maf serious:** Wt- or K33R-c-Maf were cloned into pEGFP-N1 backbone by *EcoRI-EcoRI* sites.

- **pEYFP-C1-PIAS1:** Wt-PIAS1 was cloned into pEFP-C1 backbone by *SalI-EcoRI* sites.

- **pEYFP-C1-Ubc9:** Wt-Ubc9 was cloned into pEYFP-C1 backbone by *BamHI-EcoRI* sites.

- **pGex-4T1-c-Maf:** Wt-c-Maf was cloned into pGEX-4T1 backbone by *EcoRI-Sal I* sites.

- **pGFP-RV-c-Maf serious:** Wt- K29R- K33R-c-Maf were cloned into GFP-RV backbone by *Bgl II/Bam HI-Sal I/Xho I* ligation, respectively.

- **pGilda c-Maf N-138:** N-terminal 138 base pairs of c-maf AD was cloned into pGilda by *EcoRI and XhoI* sites.

- **pcDNA3.1 HA-c-Maf serials:** Wt, K29R, K33R, K328R, K29,33,328R were cloned into pcDNA3.1 His B backbone by *EcoRI-EcoRI* sites, and then replaced the His-tag to the HA-tag by *Hind III-Kpn I* sites.

- **p3X-Flag-CMV-14-UBC9:** This vector was kindly provided by Dr. Hung-wen Chen. Wt-Ubc9 was cloned into p3XFlag-CMV-14 backbone by *EcoRI-BamHI* sites.

Part 2. Methods:

2.2-1 Immunoprecipitation assay

For the co-immunoprecipitation assay, primary Th2 cells were re-stimulated with anti-CD3 antibody for 24 hours and then lysed with NP-40 lysis buffer (50 mM Tris (pH7.4), 150 mM NaCl, 1% NP-40) supplemented with 20 mM NEM (Sigma, St. Louis, MO), PMSF (Sigma) and a 1% protease inhibitor cocktail (Sigma). Five-hundred μg cell lysate was incubated with anti-c-Maf antibody (clone M-153, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C on a rotor over night before protein G sepharose beads (Millipore) were added and incubated for another 1 hour. The beads were washed five-times in ice-cold lysis buffer and the supernatant was carefully removed. The immunoprecipitates on the beads were eluted and analyzed with Western blotting.

To detect endogenus SUMO-modified c-Maf, primary $CD4^+$ T cells obtained from BALB/c mice were cultured under a Th2- or Th17-skewing condition. Four days later, the cells were re-stimulated with anti-CD3 antibody (1 μg/ml, clone: 2C11, BioLegend, San Diego, CA) for 18 hours then were collected and lysed by NP-40 lysis buffer containing 20 mM *N*-ethylmaleimde (NEM; Sigma), the deSUMOylation inhibitor. Five-hundred μg of cell lysate of Th2 or Th17 cell was incubated with anti-c-Maf (clone M-153, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-SUMO-1 (clone D-11 , Santa Cruz Biotechnology, Santa Cruz, CA) antibodies at 4°C for 9 hours before protein G sepharose beads (Millipore) were added and incubated for another 1 hour. The beads were washed five times in ice-cold np-40 lysis buffer and the supernatant was carefully removed. The immunoprecipitates were blotted with anti-c-Maf and anti-SUMO

antibodies, respectively, or vice versa.

2.2-2 Intracellular cytokine staining

Naive $CD4^+$, $CD62L^{high}$ T cells were purified by sorting, then activated with anti-CD3 (1 μg/ml; clone 2C11; BioLegend, San Diego, CA)/ anti-CD28 (1 μg/ml; clone 37.51, e-Bioscience) antibodies (Th0-skewing condition) and transduced with GFP-RV c-Maf thirty hours later. Cells were then cultured at 37°C, in 5% CO2 for another 3 days, and re-stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml; Sigma), ionomycin (1 μg/ml; Sigma) and treated with monesin (2mμg/ml; Sigma) for 4 hours. Cells were subsequently fixed in paraformaldyhyde (4%; Sigma) and permeabilized in saponin (0.1%; Sigma). Treated cells were then incubated with anti-cytokine antibodies for 30 min on ice. Finally, cells were washed and analyzed by flow cytometry.

2.2-3 *In vitro* Th cell differentiation

Primary $CD4^+$ T cells were purified from lymph nodes with an EasySep CD4 selection kit (StemCell technologies, Vancouver, BC) according to the manufacturer's instructions. On day 0, purified $CD4^+$ T cells were harvested and stimulated *in vitro* with anti-CD3 (1 μg/ml; BioLegend, San Diego, CA)/ anti-CD28 (1 μg/ml; BioLegend) antibodies, anti-IL-4 and anti-IFNγ (clone XMG1.2; BioLegend) blocking antibody at 10 μg/ml under Th0 or Th17 skewing conditions. Recombinant IL-2 (100 units/ml; ProSpec, Rechovot, Israel) was added at days 1 and 3 to maintain cell survival and proliferation.

2.2-4 *In vivo* and *in vitro* SUMOylation assay

HEK293T (2×10^5) or DO11.10 cells (2×10^6) were transfected with different combinations of pEGFP-c-Maf or pHA-c-Maf, pEGFP-SUMO-1-GG (active form), pEGFP-SUMO-1-AA (inactive form), pUbc9-FLAG (WT), pUbc9-FLAG-C93S (catalytic site mutant) or pEF1α-SENP1-3xFlag (WT). Cells were lysed 24 hours after transfection with NP-40 lysis buffer (50 mM Tris (pH7.4), 150 mM NaCl, 1% NP-40) supplemented with 20 mM NEM, PMSF and a 1% protease inhibitor cocktail. SUMOylated-c-Maf was immunoblotted with anti-c-Maf antibody. For the *in vitro* SUMOylation assay of c-Maf, different combinations of GST-c-Maf, SAE1/SAE2 (SUMO-E1), GST-Ubc9 (SUMO-E2), GST-SUMO-1, GST-SUMO-2 or GST-SUMO-3 proteins and ATP were incubated in reaction buffer at 30°C for 1 hour. Reaction products were analyzed using immunoblotting with anti-c-Maf antibody.

2.2-5 Luciferase assay

Combinations of HA-WT- or mutated-c-Maf, EGFP-SUMO-1 GG or AA mutant, Flag-Wt or mutant SENP-1, IL-4 or IL-21 promoter luciferase reporter and Renilla vectors were co-transfected into HEK293T cells. Twenty-four hours after transfection, cells were collected and analyzed with the Dual-Glo Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activities were normalized against Renilla luciferase levels.

2.2-6 Protein stability assay

HEK293T cells (3×10^6) were transfected with WT- or K33R-HA-c-Maf only or combined with EGFP-SUMO-1-GG on 9-cm-dishes, respectively. Twenty-four hours after transfection, cells were sub-cultured in 12-well-culture plates and treated with 20 ng/ml cycloheximide (CHX; Sigma). Protein samples were collected after CHX treatment for indicted periods of time and immunoblotted with anti-c-Maf and anti-actin antibodies (Millipore). Band intensities of c-Maf and actin were calculated and the relative level of c-Maf at each time point was normalized against the actin protein level.

2.2-7 Quantitative real-time PCR

Naive $CD4^+$, $CD62L^{high}$ primary T cells were sorted, and then activated with anti-CD3 (1 μg/ml; clone 2C11; BioLegend, San Diego, CA) and anti-CD28 (1 μg/ml; clone 37.51, e-Bioscience) antibodies (Th0-skewing condition). Cells were transduced with WT or mutated GFP-RV c-Maf thirty hours after activation and then cultured for another 3 days. GFP^+ cells were sorted and re-stimulated with phorbol 12-myristate 13-acetate plus ionomycin for 4 hours. Cells were collected and their RNA were extracted by using the TurboCapture mRNA kit (Qiagen) and the cDNA was further synthesized by SuperScript II reverase transcriptase (Invitrogen). Quantitative real-time PCR was performed by using iCycler Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).

2.2-8 Retroviral constructs and transduction

The WT- and the SUMO acceptor site mutated-c-Maf were cloned into GFP-RV vectors by *BglII/BamHI-SalI/XhoI* sites. In retroviral transduction experiments, Naive $CD4^+$, $CD62L^{high}$ T cells were purified by sorting, then activated with anti-CD3 (1 μg/ml; clone 2C11; BioLegend, San Diego, CA) and anti-CD28 (1 μg/ml; clone 37.51, e-Bioscience) antibodies under neutral condition. The activated cells were then treated with 200 U/ml of IL-2 on day 0 and then retrovirally tranduced thirty hours later with the Mock- or c-Maf-carrying retrovirus.

2.2-9 Statistical analysis

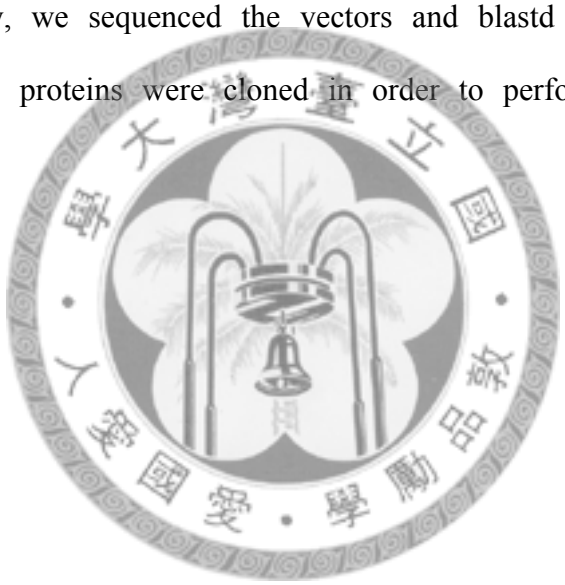
Statistical analysis was performed using an unpaired Student's *t* test. The p value was calculated and the degrees of significant difference are indicated by the number of stars (*). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

2.2-10 Yeast two-hybrid assay

Yeast-two-hybrid co-transformation system was used in our experiment. The overview of our yeast-two-hybrid co-transformation assay is shown in figure 2. Briefly, the 'baits', N-terminal 138 bp of c-Maf, was cloned into a plasmid pGilda by *EcoRI* and *XhoI* site and then transferred into a yeast strain, EGY191, which contained the Lac. Z reporter plasmid, pSH18-34. Then the 'prey', a library of plasmid pJG4-5 expressing cDNAs from the activated Th1 CD4 T cells, was transformed. The transformants that

contain the bait, lac Z reporter and the library were screened by the selection plate.

Only the colonies which contain the interacting proteins of c-Maf could survive on the selection plate. The selected colonies were picked and then performed β-gal colony-lift filter assay for the double selection. The remained clones had been re-confirmed by selection plate and β-gal colony-lift filter assay. The vectors carring cDNA library inside the candidate colonies were extracted and then grouped according to the insert size and digestion patterns by *Alu I*. This process could allows us to exclude sister clones. Finally, we sequenced the vectors and blastd the encoding genes. Full-length candidate proteins were cloned in order to perform the subsequently experiments.



Chapter III Results

3.1 Screening the c-maf interacting proteins

We performed the yeast two-hybrid (Y2H) assay to identify the c-Maf interacting protein(s). First, we cloned the "Bait" construct for our experiment. The AD region before MTD (138 bp) was chosen as the bait to avoid auto-trigger the reporter genes, and the Th1 CD4 cDNA library was used as 'Prey" to perform the Y2H co-transformation screening (Figure 1B). Nine hundred fifty-four colonies were picked up and tested the by survival and lac. Z lift assay. After rechecked by selection dish and the lift assay, only 90 candidates were selected for further investigated. The prey plasmid of the candidates were extracted from yeast and the unknown cDNA inserts were amplified by PCR and grouped by their cDNA insertion size. Similar sized candidate members were further classified by Alu I digestion analysis. The non-repeated candidates were narrowed down to 23 groups, and then sent for sequencing.

3.2 c-Maf interacts with PIAS1 and Ubc9

Table 1 is the list of selected c-Maf interacting candidates. Only three candidate's protein sequences were in-frame, and they were Ubc-9, PIAS1 and synectin. Synectin could not trigger the reporter genes when we tested it again. So we were focus on UBC-9 and PIAS1 in the following studies.

First, we examined whether the c-Maf co-localizes with Ubc9 and PIAS1. DsRed-c-Maf, EYFP-Ubc9 and EYFP-PIAS1 fusion protein vectors were constructed

and introduced into HEK293T cells. Fluorescence microscopy showed that c-Maf co-localized with Ubc9 and PIAS1 inside the nucleus (indicated by DAPI staining) (Appendix Figure 1). These data indicate that c-Maf might physically interact with Ubc9 and PIASI in the nucleus.

Next, we examined whether c-Maf could interact with the SUMO-conjugating enzymes in primary Th cells. Naïve $CD4^+$ T cells were cultured under Th2-skewing condition and re-stimulated with anti-CD3 antibody. The physical interaction between c-Maf and PIAS1 was examined with co-immunoprecipitation. We found that PIAS1 co-precipitated with anti-c-Maf antibody but not with control rabbit IgG antibody (Figure 2), demonstrating their physical interaction in primary Th2 cells. The interaction between c-Maf and PIAS1 was also confirmed by the overexpressing experiment in HEK293T cells (Data not show). We could not co-precipitate the c-Maf and Ubc9 in primary T cells.

3.3 c-Maf is SUMOylated both *in vitro* and *in vivo*

Does the c-Maf be SUMOylated by Ubc9 and PIAS1? It is known that SUMOylation generally targets the lysine residue of the consensus ΨKXE motif. In search of the consensus SUMO site(s) of c-Maf using ELM software (http://elm.eu.org/), we found three putative SUMO conjugating sites: lysine 29 and 33 residues in the AD and lysine 328 in the b-zip domain (Figure 4A). Interestingly, the first two putative SUMO accepter sites located within the region that we used as the "Bait" on Y2H assay.

We first set to study whether c-Maf could be modified by SUMOs with both *in vitro*

and *in vivo* SUMOylation assays. For the *in vitro* SUMOylation assay, purified GST-tagged c-Maf protein was co-incubated with SAE1/SAE2 (SUMO E1) and Ubc9 (SUMO E2) in the presence or absence of GST-SUMO-1, -SUMO-2 or -SUMO-3. Figure 3 shows that in the absence of SUMO, only one protein band of approximately 70 KD, which representing the unmodified GST-c-Maf, was detected. However, in the presence of SUMO, higher molecular bands were detected by c-Maf antibody. The major one was of 120 KD, corresponding to mono-SUMOylated-c-Maf. In the presence of SUMO-2 or SUMO-3, another 170 KD band was detected. This band is probably representing di-SUMOylated-c-Maf.

In vivo SUMOylation of c-Maf was performed in RK13 and in HEK293T cells. RK13 cells were cotransfected with EGFP-c-Maf and EGFP-SUMO1-GG (the active form of SUMO) combined with SENP-1 (the deSUMOylation enzyme), UBC9 (the SUMO E2) plus PIAS1 (SUMO E3) or all of them. Higher molecular band were detected when we co-expressed c-Maf and SUMO1-GG. Expression of UBC9 and PIAS1 further enhance the amount of SUMO-modified c-Maf. In contrast, SUMO-c-Maf bands were reversed when the desumoylation enzyme SENP-1 was existence. Same results were detected when we immunobloted with either anti-c-Maf or -EGFP antibodies (Fig. 4B).

In HEK293T cells, cells were co-transfected with vectors expressing HA-c-Maf and EGFP-SUMO1-GG, EGFP-SUMO1-AA (the inactive form of SUMO), Ubc9, WT SENP1 or SENP1-C603S (an inactive form of SENP1). WT-c-Maf was SUMOylated by SUMO1-GG but not by SUMO1-AA. Co-expression of SENP1-C603S, but not WT-SENP1, led to more SUMOylation of c-Maf (Figure 4C, left panel). These results indicated that the c-Maf underwent dynamic SUMOylation and de-SUMOylation in

HEK239T cells. SENP1-C603S might function as a dominant negative mutant of endogenous SENP1, preventing the removal of SUMO from SUMOylated-c-Maf, and resulting in an accumulation of SUMOylated-c-Maf. We also generated a c-Maf mutant, K29,33,328R, in which the lysine residues within the three predicted SUMO-conjugating sites were mutated to arginine. In experiments using the mutants, we detected very little, if any at all, SUMOylated-K29,33,328R c-Maf in HEK293 cells. This data indicated that c-Maf conjugated with SUMO in one of these predicted sites (Figure 4C, right panel).

3.4 Lycine 33 is the dominant SUMO acceptor site of c-Maf

Having confirmed that c-Maf could be modified by SUMOylation, we set up to identify the SUMO acceptor site(s) of c-Maf. Three c-Maf mutants, K29R, K33R, and K328R, were generated by replacing the lysine at positions 29, 33, and 328 to arginine. To test which mutant could not conjugate with SUMO, each mutant was transfected into HEK293T cells to perform the *in vivo* SUMOylation assay. Both K29R- and K328R-cMaf were modified by SUMO as WT-c-Maf was and that K33R-c-Maf completely resistant to SUMOylation (Figure 5A). These results indicated that K33 is the dominant, if not the only, SUMO acceptor site of c-Maf. To confirm that K33 is also the SUMO acceptor site of c-Maf in T cells, we expressed either WT or K33R-c-Maf in DO11.10 cells. *In vivo* SUMOylation analysis showed that K33R-c-Maf did not undergo SUMO modification in DO11.10 T cells (Figure 3B). The results of *in vitro* and *in vivo* assays demonstrate that lysine at position 33 but not other position is the SUMO acceptor site.

3.5 SUMOylation attenuates c-Maf transactivity

SUMOylation was reported to modulate activities of many transcription factors. Whether the c-Maf is affected by SUMO? We preformed the luciferase assy to address this issue. HEK293T cells were transfected with 2X MARE-promoter luciferase reporter, combined with Wt-, K29R, K33R or K328R-c-Maf encoding vectors. The results showed that both K29R- and K328R-cMaf have similar transactivities as Wt-c-Maf, but the SUMO deficient K33R-c-Maf has significantly higher (~50%) activity to transactive MARE promoter luciferase (Figure 6A.). Next, we use the *Il4* promoter-leuciferase reporter to test the effect of Ubc9 and PIAS1 on c-Maf transactivation abilty. As our prediction, both Ubc9 and PIAS1 attenuate the *Il4* promoter transactivity of c-Maf in a dose dependent manner (Figure 6B).

Finally, we investigated the SUMOylatin effects on Wt and its SUMO mutant by cotransfected Wt- or K33R-c-Maf with SUMO-1 GG, SUMO-1AA or SENP-1 into HEK293T cells. As shown in Fig. 6C, only Wt-, but not K33R-c-Maf was affected by SUMO-1 GG or SENP-1. The K33R mutant showed higher (approximately 1.5 fold) Il4 promoter transactivity than the Wt-c-Maf. SUMO-1 mutant (SUMO-1 AA) has no effect on both Wt or K33R c-Maf. Wild-type SENP1 enhanced the transactivation ability of Wt c-Maf, but not the K33R mutant. These data indicated that K33 (VKKE) is important for c-Maf function. Sumoylation of c-Maf on lycine 33 will impair its ability to transactivate IL-4 (Figure 6C).

3.6 SUMOylation of c-Maf in Th2 clone and primary Th2 cells

We further examined whether SUMOylation of c-Maf occurred in Th2 cells. The Th2 clone, D10 cells were stimulated with PMA/ionomycin for four hours and lysed. Cell lysates were immunopericipated with anti-c-Maf antibody and immunoblotted with anti-c-Maf antibody. Higher molecular band (approxmatily 70KD) of c-Maf was detected (Fig. 7A).

We then employed primary $CD4^+$ T cells to study whether c-Maf is SUMOylated under Th2 condition. Primary Th2 cells were generated *in vitro* and restimulated before harvest. Cell lysate was prepared and subjected to immunoprecipitation with anti-c-Maf, anti-SUMO-1, or control antibody. Endogenous non-SUMOylated-c-Maf (approximately 50 KD) was immunoprecipited and detected by anti-c-Maf antibody (Figure 7B, top panel). Anti-c-Maf also precipitated a much less abundant band of 70 KD, approximately the size of SUMOylated-c-Maf, that was recognized by both anti-c-Maf and anti-SUMO-1 antibodies (Figure 7B, top and middle panels). Furthermore, anti-SUMO-1 antibody also precipitated a 70 KD protein that was recognized by anti-c-Maf antibody (Figure 7B, bottom panel). These results demonstrate that c-Maf is SUMOylated not only in HEK293T cells but also in re-activated primary Th2 cells under physiological conditions.

3.7 SUMOylation suppresses c-Maf-dependent *Il4* gene expression

Previous studies have shown that c-Maf serves as a key regulator of IL-4 production (Ho et al., 1996; Kim et al., 1999). The next question was whether SUMOylation had

any impact on the c-Maf-dependent *Il4* gene expression. To determine whether the effect of SUMOylation on c-Maf could also be observed in T cells, we performed the *Il4-promoter* luciferase assay on T cell line DO11.10. The results showed that the c-Maf activity in T cells was also attenuated by SUMO (p=0.002) and enhanced by SENP1 (p=0.035) (Appendix Figure 2A). Furthermore, the K33R-c-Maf in T cells was still more potent than the WT-c-Maf in transactivating the *Il4-promoter* (p=0.024) and resistant to the effect of SENP1 (p=0.019) (Appendix Figure 2B).

To examine the effect of c-Maf SUMOylation on the endogenous *Il4-promoter*, primary $CD4^+$ T cells taken from wild-type BALB/c mice were activated and skewed under Th0 condition, and then transduced with retroviral vectors expressing GFP alone or with either WT- or K33R-c-Maf. The level of cytokines production by the transduced cells was measured by quantitative real time-PCR or with intracellular cytokine staining. Comparing to the Mock retroviral transduced GFP+ cells, the Wt-c-Maf transduced GFP+ cells showed the IL-4 mRNA expression and more IL-4-producing cells (increase from 10.3% to 15.9%) under the same skewing condition. Furthermore, the K33R mutant transduced cells showed approximately 3-fold higher IL-4 mRNA expression and enhanced about 20% of IL-4-producing cells (increase from 15.9% to 19.1%). The MFI of K33R mutant transduced cells were also higher than the Wt-c-Maf transduced cells (increase from 76.1 to 81.8) (Figure 8A and 8B).

To rule out the effect of endogenous c-Maf, we further employed c-Maf-deficient primary Th cells to repeat the experiment. Lymph node $CD4^+$ T cells were taken from RAG2 knockout mice that were reconstituted with c-$Maf^{-/-}$ fetal liver cells. c-Maf-/- Th cells were used to avoid any confounding effects from endogenous Wt-c-Maf. Wt and

the c-Maf mutants were separately introduced into $CD4^+$ T cells by retroviral transduction. After activation under non-skewing conditions, the cells were then transduced with a retroviral vector expressing GFP alone or in combination with either WT-, K29R- or K33R-c-Maf. The production of endogenous cytokines by the transduced cells was examined with intracellular cytokine staining. As shown in Appendix figure 3 ,expectedly, expression of WT-c-Maf enhanced the percentage of IL-4-producing cells (increase from 10% to 17.9%) and K29R mutation had no impact on the activity of c-Maf. However, K33R mutation enhanced IL-4-producing cells by about 3-fold (approximately 30%)**.** It is noteworthy that the percentage of GFP^+ $IL\text{-}5^+$ cells was also higher in the cells transfected with K33R mutant than that with the WT-c-Maf (6.31% vs. 2.97%). Although it awaits to be confirmed, the increase in IL-5 production is likely a secondary effect of higher IL-4 production. Interestingly, the percentages of $IL\text{-}2^+$ cells were comparable amongst groups that were transfected with WT-, K29R-, and K33R-c-Maf, showing that the expression of c-Maf doesn't affect the activation of *il2* gene. These data indicate that a SUMOylation resistant c-Maf is more potent than WT-c-Maf in driving endogenous IL-4 production in c-Maf-deficient Th2 cells (Appendix Figure 3).

3.8 DeSUMOylation enhances the recruitment of c-Maf to the *Il4-promoter*

How does SUMO modification affect the function of c-Maf? To address this question, we tested the protein stability, cellular localization and the recruitment of c-Maf to the *Il4* promoter MARE site. We first examined whether c-Maf protein stability is altered by SUMOylation. WT- or K33R-c-Maf alone or combined with SUMO-1 were expressed in HEK293T cells for one day and the cells were treated with cyclohexamide (CHX) to

inhibit protein synthesis. Cells were collected at different time points after CHX treatment and the changes of c-Maf protein levels were measured. As shown in Figure 9A, the half-lives of wild type and its K33R mutant were not statistically different from each other. The stability of Wt- and K33R-c-Maf was also similar when SUMO molecules were added (Figure 9B). These results indicated that SUMO modification does not alter the protein stability of c-Maf.

Next, the subcellular localization of Wt-c-Maf and its K33R mutant was then analyzed. pEGFP-WT- or pEGFP-K33R-c-Maf fusion protein was expressed in HEK293T cells to compare their cellular localization. Confocal imaging showed that both WT-c-Maf and K33R-c-Maf were located exclusively in the nucleus (Figure 10A). Furthermore, the exclusive cellular localization of c-Maf was not altered by overexpression of SUMO molecules. Both c-Maf and SUMO co-localized within the nucleus when overexpressed in HEK293T cells (Figure 10B). These data demonstrate that SUMOylation doesn't alter the subcellular localization of c-Maf.

The third question was whether SUMO modification affects c-Maf recruitment to the endogenous *Il4-promoter*. DO11.10 cells expressing either EGFP-WT-c-Maf or EGFP-K33R-c-Maf were subjected to chromatin immunoprecepitation (ChIP) assay. Western blotting revealed that a protein of molecular weight equivalent to SUMOylated-c-Maf was present in DO11.10 cells expressing WT-c-Maf but not in cells expressing K33R-c-Maf (Appendix Figure 5A). The CHIP assay results showed that both original and SUMOylated c-Maf could recruit to the *Il4* promoter. Interestingly, while both WT- and K33R-c-Maf were recruited to the *Il4-promoter*, the recruitment of SUMO deficient K33R-c-Maf was four-times more robust than that of the WT-c-Maf

(Appendix Figure 5B). The result strongly indicates that SUMOylation at the K33 site inhibits the activity of c-Maf by attenuating its recruitment to the *Il4-promoter*.

It has been reported that SUMOylated proteins could be accumulated within the PML-NBs. Recently, c-Maf was reported to recruit into the PML-NBs (Hiramatsu et al.). We also observed that Wt-c-Maf, but not its K33R-mutant, could form the spots within the nucleus (Figure 16). Whether these spots are the PML-NBs should be further addressed,

3.9 c-Maf, but not other transcription factors, transactivates Il-21 gene expression

Previous study has showed that the deficiency of c-Maf results in reduced IL-21 production in Th17 cells (Pot et al., 2009). It is possible that c-Maf acts as a transcription factor for IL-21 gene expression. First we analyzed the *il21* promoter and found four putative c-Maf binding sites on IL-21 proximal promoter. Four putative MARE sites located in -1070 bp (half MARE), -370 bp (v-MARE), -249 bp (half MARE) and -200 bp (v-MARE) upstream of the transcriptional start site, respectively. The localization and their sequences are shown in Fig. 12A.

To test whether the IL-21 gene expression can be activated by c-Maf, we performed the luciferase assay to address this issue. Luciferase reporter containing the *il21* promoter (from -1324 to +45 of *il21* promoter) was cloned for the following studies. First we tested whether the c-Maf is the specific transcription factor for IL-21 gene regulation. The IL-21 promoter-luciferase reporter (-1324 to +45) combined with different transcription factors were cotransfected into HEK293T cells and to see if any

transcription factors could induce IL-21 gene expression. We found that only the c-Maf, but not other transcription factors (such as the T-bet, GATA3, T-bet, Foxp3, BCL6 or RORγt) transactivated IL-21 promoter-luciferase (Fig. 12B).

Next, we cotransfected the *il21* promoter luciferase reporter with different concerntrations of c-Maf expression vector into HEK 293T cells. We found that c-Maf transactivate IL-21 promoter-luciferase in a dose-dependent manner (Fig. 12C). These results suggesting that c-Maf is the IL-21 gene specific transcription factor.

3.10 c-Maf transactivates IL-21 expression through proximal MARE site

Next we tried to define c-Maf-binding site(s) within the IL-21 promoter. Several IL-21 promoter-luciferase reporter vectors were made by truncating the promoter or mutating the MARE sites sequence of *il21* promoter. We generated the -342 *il21* promoter reporter vector which was truncated the -1070 and -370 MARE sites. Based on the -342 *il21* promoter reporter vector, the -342m249 reporter mutated the -249 MARE site and the -342m200 reporter vector mutated the -200 MARE site were generated. We also made the -229 *il21* promoter reporter vector which deletted the -1070, -370 and -249 MARE site. And the -229m200 reporter vector mutated -200 MARE site was generated. The list of these reporter constructs were shown in Fig. 13.

To find out the c-Maf binding site(s), we compared the responsiveness of these reporters to c-Maf. The -1324 promoter reporter, the -342 promoter reporter and the -229 promoter reporter could response to the c-Maf comparing to the promoter reporter only. Only the -342m200 promoter reporter and the -229m200 promoter reporter

showed no induction fold by c-Maf. According to these luciferase assay results, we concluded that -200 MARE site of IL-21 promoter was essential for c-Maf to transactivating the IL-21 gene expression. (Appendix Figure 4)

3.11 c-Maf is SUMOylated in Th17 cells

We and other group have sown that c-Maf could be SUMOylated in Th2 cells and negatively regulated the c-Maf dependent Il-4 gene expression (Leavenworth et al., 2009; Lin et al.). We further tested whether the c-Maf is also been SUMOylated in Th17 cells and affects IL-21 gene expression.

Primary Th17 cells were generated *in vitro* and restimulated with anti-CD3 for 18 hours. Cell lysate was prepared and subjected to immunoprecipitation with anti-c-Maf, anti-SUMO-1, or control antibody. Endogenous non-SUMOylated-c-Maf (indicated by arrows) was detected (Figure 14A, top panel). Anti-c-Maf also precipitated a much less abundant band of 70 KD, approximately the size of SUMOylated-c-Maf, that was recognized by both anti-c-Maf and anti-SUMO-1 antibodies (Figure 14A, top and middle panels). Furthermore, anti-SUMO-1 antibody also precipitated a 70 KD protein that was recognized by anti-c-Maf antibody (Figure 14A, bottom panel). Taken tougher, c-Maf could undergo SUMOylation in Th17 cells as well as in Th2 cells.

3.12 SUMOylation deficient K33R c-Maf has more potent IL-21 gene transactivity

To test whether the of SUMOylation affects c-Maf to drive the IL-21 expression. HEK293T cells were transfected with an *il21* promoter (-229) luciferase reporter vector,

Wt- or K33R-c-Maf expression vector, and/or SENP1 expression vector. Figure 11 showed that c-Maf transactivated the *il21* promoter by nearly 2-fold as compared to vector alone. K33R-c-Maf showed higher transactivity than Wt-c-Maf. In addition, SENP1 enhanced Wt-c-Maf-induced *il21* promoter activity. K33R-c-Maf slightly increased in its induction fold (Figure 14B)**.** These data indicate that SUMO deficient c-Maf is more potent to transactivate the *il21* promoter.

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Chapter IV Discussions

In this report, we characterized that c-Maf is post-translational modified by SUMO molecules. We identified the lysine 33 is the only acceptor site for SUMO. Like most other SUMO modified proteins, conjugating with SUMO suppresses the transactivity of c-Maf. In addition, retroviral transduction with SUMO-deficient K33R-c-Maf induces more IL4 production than wild type-c-Maf does in primary c-Maf-deficient T cells. Moreover, the SUMO-deficient c-Maf shows higher recruitment to the *Il4* promoter. In conclusion, our data suggest that SUMOylation modification acts as a negative regulator of IL-4 production induced by c-Maf through attenuates the recruitment of c-Maf to the *Il4* promoter.

In order to perform the appropriate biochemical response, the activity of transcription factor should be tightly regulated. One major way is through the post-translational modification. Previous studies on the post-translational modification of large Maf proteins were focused on their phosphorylation. Phosphorylation is the most common, rapid and important modification of proteins. c-Maf has been reported to be phosphorylated by P38 and GSK-3 (Sii-Felice, 2005; Rocques, 2007). The large Maf proteins, MafA, MafB and c-Maf, were reported to be phosphorylated by P38 MAP kinase (Sii-Felice, 2005). Recently, sequential phosphorylated of MafA by GSK3 on S61, T57, T53 and S49 was found. The phosphorylation of MafA enhanced its ubiquitination and increased the recruitment of coactivator P/CAF by MafA (Rocques, 2007). In this paper, c-Maf was reported to be phosphorylated by GSK-3 in two human multiple myeloma cell lines. These studies pointed out that the large Maf family has

been post-translational modification by serine/threonine phosphorylation.

The activation domain (AD) is important for the activity of c-Maf. Many modifications are resided in this region. Acidic region was reported to undergo Serine/threonine phosphorylation by P38 MAP kinase and GSK3 (Sii-Felice, 2005; Rocques, 2007). Two conserved serine residues in acidic domain are required for c-Maf mediated CD13 expression in activated endothelial cells (Mahoney, 2007). The AD region is also important for the protein-protein interactions with other partners of c-Maf. The coactivators, CBP/P300, were reported to interact with c-Maf AD domain and lead to up-regulation of crystallin gene expression (Chen, 2002). Large Maf proteins also interact with TATA-binding protein through the minimal transactivation domain (MTD) inside the acidic domain (Friedman, 2004). Here we showed that c-Maf could be SUMOylated in lysine 33 which locates right before the MTD (amino acid 47~110). Furthermore, these modification and interaction are broadly existing in large Maf proteins, suggesting that the acidic domain of large Maf proteins are not only critical for their functions but also important in controlling the protein activities by multiple post-modification and recruitment of interacting proteins.

Recently, cross-talks between different post-translational modifications were identified. The cross-talk could be held between neighboring amino acid residues. The proteins containing the phosphorylation-dependent SUMOylation motif (PDSM), such as heat shock factors 1 (HSF1) and PPARγ, can perform the SUMOylation/phosphorylation switching (Hong, 2001; Yamashita, 2004). Moreover, the same lysine residue of protein can be subjected to multiple post-translational modifications (Freiman, 2003). For example, IκBα use the same lysine residue for its

SUMOylation and ubiquitination (Desterro, 1998). Further study indicated that SUMOylation of IκBα is required for its subsequently ubiquitination (Cheng, 2007). In addition, HIC1 and C/EBPδ which contains the ΨKXEP motif were reported to be modified by either actylation or SUMOylation at the same lysine residue (Stankovic-Valentin, 2007; Wang, 2006). Moreover, MEF2D which contains the ΨKXEPXSP motif can undergo the much more complicated switching between acetylation,SUMOylation and phosphorylation (gregoire, 2006; gregoire, 2005). In the case of c-Maf, the amino acid sequences neighboring the residue lysine 33 show no characteristic of PDSM but match the ΨKXEP motif that favors the actylation/SUMOylation switching event. Since the acetylation of c-Maf has not yet been studied, whether the lysine 33 residue of c-Maf could undergo acetylation and further engage in the switching with SUMOylation will be interesting topics.

After the finding the SUMO molecules, many studies focused on whether the protein interested could be modified and regulated by SUMO. MafB and MafA were reported to be modified by SUMO (Tillmanns et al. 2007; Shao et al. 2008). MafA was conjugated by SUMO-1 and SUMO-2 on residue lysine 32 which is induced by hypoglycemia or by oxidative stress. MafB was reported to be SUMOylation by SUMO-1 on lysine 32 and 297 residues via v-myb signaling. Here we first reported that c-Maf undergoes SUMOylation on lysine 33 residue. This observation is supported by abolishing SUMOylation on mutant c-Maf that lysine 33 is changed to arginine. The SUMO acceptor site of c-Maf just located on the N-terminal region that we used as a bait to perform the yeast two-hybrid study. By the yeast two-hybrid screening, we identified that SUMO E2 and E3, Ubc9 and PIAS1 respectively, interact with c-Maf. Large Maf proteins share two interisting characteristics on their SUMO modification. First, all large

maf proteins use the consensus site on activation domain for their SUMOylation, ie. lysine 33 of c-Maf, lysine 32 of MafA and MafB (Figure 15). Second, the effects of SUMOylation on these large Maf protein functions were all negative. Sumoylated MafA decreased its transactivity on insulin promoter and then enhanced the suppression of CHOP10 expression (Shao, 2008). Moreover, SUMOylation suppressed MafB to promote macrophage differentiation and inhibited the cell cycle procession (Tillmanns, 2007). Our results also showed that SUMO negatively regulate Il4 gene expression. Taken together, SUMOylation of conserved lysine recidue among the large Maf protein members on acidic domain seems to be the evolutional strategy to regulate the activities of large Maf proteins.

Until now, the regulation of SUMO system is still poorly understood. SUMOylation is a highly dynamic process. The overall SUMOylation level of target protein is the outcome of the balance between SUMO and de-SUMO process. The SUMO system can be regulated by controlling the expression of SUMO or de-SUMO enzymes, the activities of SUMO enzymes or the competition from other post-translational modification (Liu, 2008). Calcium influx was reported to induce the SUMO E1, E2, PIASx and SUMO2/3 expression in keratinocyte (Deyrieux, 2007). In contrast, IL-6 was reported to induce deSUMOylation through gp130 signaling by induction the deSUMOylation enzyme SENP1 (Cheng, 2007; Ohbayashi, 2008). Interestingly, previous study has shown that IL-6 up-regulates c-Maf transcription independent of IL-4/STAT6 signals, but through the binding to the c-maf promoter by STAT3 in CD4 T cells (Yang, 2005). Whether the induction of SENP-1 by IL-6 also facilitates c-Maf expression should be further examined.

Besides c-Maf, IL4 production is also controlled by different transcription factors binding to its promoter, such as NFAT, AP-1, C/EBPβ and YY1 etc (Li-Weber, 2003). Some of these transcription factors were reported to be SUMOylated, but the effects of SUMOylation on these factors are quite different. For example, JunB, the AP-1 family member, was shown to be SUMOylated on residue lysine 237 residue in T cells. Blocking JunB SUMOylation diminished JunB mediated IL-2 and IL-4 gene expression (Garaude, 2008). The CCAAT/enhancer-binding protein beta (C/EBPβ) was reported to transactivate IL-4 gene expression in murine T cells and facilitates Th2 cell responses. Its SUMOylation inhibited C/EBPβ mediated repression of c-myc expression. However, SUMOylation did not affect its activity on IL4 gene expression (Berberich-Siebelt, 2006). The Th2 master regulator was reported to interacte with Ubc9 and PIAS1. Overexpressing PIAS1 enhanced GATA3 driven IL-13 expression but not affect IL-4 production (Zhao, 2007). NFAT and YY1 were also documented to be SUMO modified, but the effect of SUMO modification on the IL4 production have not yet been addressed (Terui, 2004; Deng, 2007). Although several transcription factors involved in IL-4 gene expression, but the overall effect on IL-4 production on these SUMO-conjugating factors remains unclear.

Certain transcription factors that regulate IL-21 gene expression have been identified. NFAT was reported to bind directly to the *il*21 promoter and then promoted the synthesis of IL-21 production in Th2 cells (Kim et al., 2005; Mehta et al., 2005). IRF-4 was also found to drive IL-21 expression by binding to *il*21 promoter (Chen et al., 2008). On the other hand, IL-6 and IL-21 can activate the STAT3 and further induced IL-21 production in Th17 cells (Nurieva et al., 2007). Recently, the AP-1 family protein Batf was reported to play critical roles in Th17 differentiation, which binds to *il17*, *il21* and

il22 promoters and transactivates the expression of these cytokines expression(Betz et al., 2010; Schraml et al., 2009). Furthermore, c-Maf was found to be highly expressed in Th-17 cells and Tfh cells and played an important role in IL-21 production of Th-17 and T_{FH} cells (Bauquet et al., 2009). Recent study showed that IL-21 production in Th17 cells in reduced in c-Maf deficient T cells (Pot et al., 2009). It is interesting to investigate the regulating networks of these IL-21 transactivaters in Th17 and Tfh cells.

More and more proteins were reported to be SUMOylated and their functions were modulated by SUMOylation. Are these IL-21 transactivators could be SUMOylated? NFAT was reported to be modified by SUMO, but the effect of SUMOylation on NFAT mediated IL-21 production remains unclear. There are no study reported that IRF-1 and Batf could be SUMOylated. SUMOylation of STAT3 was not yet clear, and STAT3 has no consensus SUMO acceptor site in its protein sequence. However, the SUMO E3 ligase PIAS3 could interacts with STAT3, and that STAT3 was observed to be recruited into PML-NBs (Ohbayashi et al., 2008), suggesting that STAT3 might also be SUMOylated. Whether these transcription factors undergo SUMOylation could be further studied. Here, we first showed that c-Maf also be SUMOylated in Th17, as well as in Th2, and plays as a negative regulator for c-Maf-dependent IL-21 gene expression. All the large Maf proteins were reported to be SUMOylated on consensus SUMO accepter site and suppressed their activities, indicating that the SUMOylation is a conserved regulatory mechanism for Large Mafs.

Unlike the atypical half MARE site on *Il4* promoter (Ho et al., 1996), the c-Maf binding site on *il21* promoter is the typical v-MARE sequence. Notably, the induction fold of *il21* promoter-luciferase reporter driving by c-Maf was quite low than the *Il4*

promoter does, suggesting that some essential factor which cooperates with c-Maf for IL-21 transactivation was insufficient in our luciferase assay. Maf protein binds to the MARE by forming the homodimer, or forming the hetrodimer with other Mafs or with AP-1 family members. On the other hand, NFATc2 and c-Maf have been reported to synergistically transactivate the *Il4* promoter in Th2 cells (Hodge et al., 1996). Furthermore, IRF4 interacts with NFATc2 to modulate IL-4 gene expression (Rengarajan et al., 2002). Interestingly, a NFAT binding site just locates close to the -200 MARE site in *il21* promoter. Whether c-Maf cooperates with AP-1 or NFAT family proteins to promote the IL-21 production could be further investigated.

Previous studies have shown that c-Maf also binds to the -206/-171 region in the *Il-10* promoter and dives the Il-10 production in monocytes and macrophages (Cao et al. 2002; Cao et al. 2005). c-maf also suppresses IL-12 receptor gene transcription. Whether the SUMOylation could affect these functions of c-Maf and what is/are the mechanism(s) underlined should be further characterized.

Post-translational SUMOylation regulates transcription factor activity through different ways, such as mediating the cellular localization, protein stability, DNA binding or protein-protein interaction (Gill, 2003; Verger, 2003). SUMOylation could affect the target proteins by altering their cellular localization. TEL and Daxx, for instance, were reported to alter their cellular localization by SUMOylation (Wood, 2003; Muromoto, 2006). We have observed that both wild type and K33R SUMO-deficient mutant are located inside the nucleus by confocol microscopy. Similarly, SUMOylation of MafA did not alter its original cellular localization (Shao, 2008). These finding implied that SUMO do not alter the localization of large Maf proteins. Moreover, many

SUMOylated proteins were demonstrated to be recruited into the subnuclear PML nuclear body (PML-NB) (Heun, 2007). Recently, recruitment of Wt-c-Maf, but not K33R SUMO deficient, within the PML-NBs was reported (Leavenworth et al., 2009). We also observed that c-Maf form spots inside the nucleus, but we did not exam whether the c-Maf was co-localized with the PML is an interesting topic.

The protein stabilities of Pax8 and GSK3β were reported to alter by SUMOylation (de Cristofaro, 2009; Eun Jeoung, 2008). However, the protein stability of MafA is not affected by SUMOylation (Shao, 2008). Similarity, wild type and SUMO-deficient c-Maf did not display statistically difference on their degradation rates in our study. Thus, we concluded that the half life of c-Maf does not change by SUMOylation. Finally, SUMOylation was documented to affect the DNA binding ability of their target proteins, for example, thymine DNA gylcosylase (TDG) and Daxx (Baba, 2005; Lin, 2006). We tested the abilities that wild type- and K33R-c-Maf interact with MARE binding sequence in vitro and their recruitment to the *Il4* promoter in vivo by EMSA and ChIP assay, respectively. There is no difference between wild type- and SUMO-deficient K33R-c-Maf interacting with MARE sequence by EMSA (data not shown). In contrast, revealed by the ChIP data, SUMO-deficient K33R-c-Maf increased recruitment to the *Il4* promoter than the wild type-c-Maf did. Thus, it explains why the IL-4 production inducing by SUMO-deficient K33R-c-Maf is higher than that by wild type-c-Maf.

What is the underlining mechanism by which enhances the recruitment of SUMO-deficient c-Maf to the *Il4* promoter? SUMOylation may attenuate c-Maf dependent *Il4* gene expression by dynamically restrict the c-Maf within PML-NBs. The

SUMO deficient K33R-c-Maf is resistant to this process, thus it has higher transactivation ability compare to the Wt-c-Maf. However, we can not exclude the existent of additional mechanisms that contribute to SUMO-dependent attenuation of c-Maf transactivity. Is the DNA binding of c-Maf to the *Il4* promoter disrupted by SUMO? Since the SUMO conjugating site of c-Maf is located on N-terminal AD region, far away from the C-terminal DNA binding region, the SUMO molecules might not directly block the c-Maf binding to the MARE sites. The data from DNA-protein pull-down assay supported that c-Maf-*Il4* promoter interplay is not affected by SUMOylation. One possibility is the c-Maf recruitment to the DNA is mediated by post-translational modification switching. Another possibility is that the SUMOylation of c-Maf alters the interaction with its partner(s). The AD region has shown to interact with TBP and CBP/P300 respectively and these interacting proteins are important for c-Maf activity (Friedman, 2004; Chen, 2002). Whether the interactions are attenuated or inhibited by SUMO conjugation on c-Maf AD should be further addressed. To date, many proteins are reported can interact with SUMO through the SIM, the SUMO interacting motif (Hecker, 2006). Protein that is modified by SUMO may interact with these proteins carrying the SIM. The transactivation complex of c-Maf may also be disrupted or altered by these SIM containing protein(s). Furthermore, some SUMOylated protein carrying the SIM itself, which can interacts with self SUMO molecule and induces the intra-molecular comformational change (Steinacher, 2005). Whether the c-Maf interacts with new partner(s) through SUMO molecule or c-Maf itself containing the SIM need to be further tested.

Taken together, we provide the evidence that c-Maf activity could be modulated by the SUMO system. We also identified the SUMO conjugating site at lysine 33 residue of

c-Maf. More importantly, it is indicated that the IL-4 and IL-21 production driving by c-Maf is significantly attenuated by SUMO-modification. How SUMOylation affects the recruitment of c-Maf will be further investigated.

Chapter V Figures and Tables

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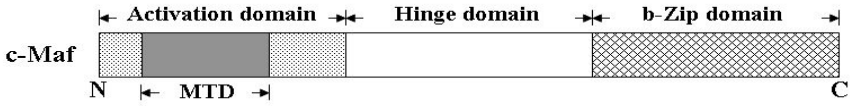

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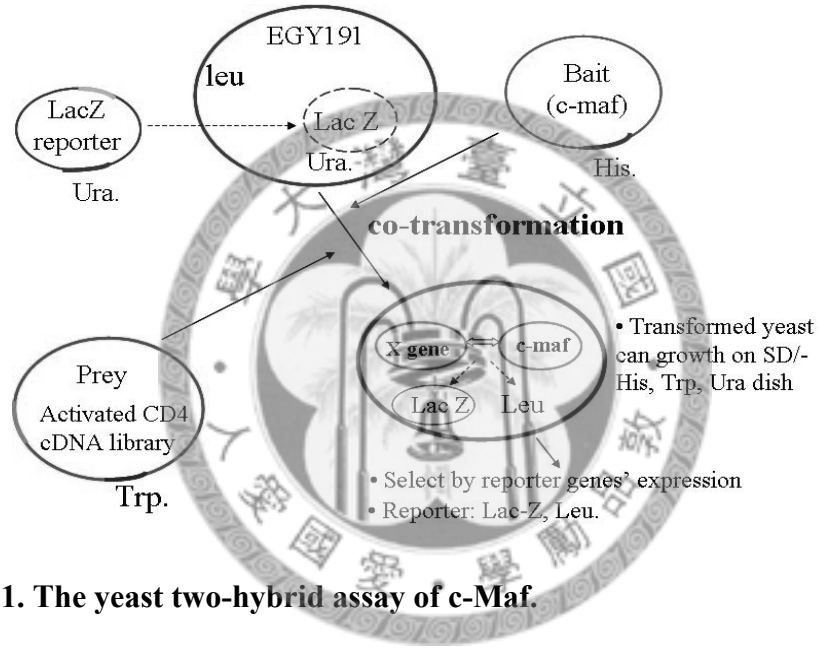

FIGURE 1. The yeast two-hybrid assay of c-Maf.

(A) Illustration of c-Maf functional domain. There are three functional domains in c-Maf. They are N-terminal Activation domain (AD), C-terminal b-Zip domain and both are linked by hinge domain. The minimal transactivation domain (MTD) locates inside of the activation domain. **(B) The co-transformation yeast two-hybrid system.** Co-transformation yeast two-hybrid system was chosen to investigate the c-Maf interacting candidates. EGY191 yeast stain and pSH18-34 Lac. Z reporter were used in this screening. The N-terminal 138 bps of c-Maf, was cloned into the pGilda vector as the "Bait" and the activated Th1 cDNA library was used as the "prey". The yeasts were transformed with bait, prey and Lac.Z vectors and selected by selecting dishs.

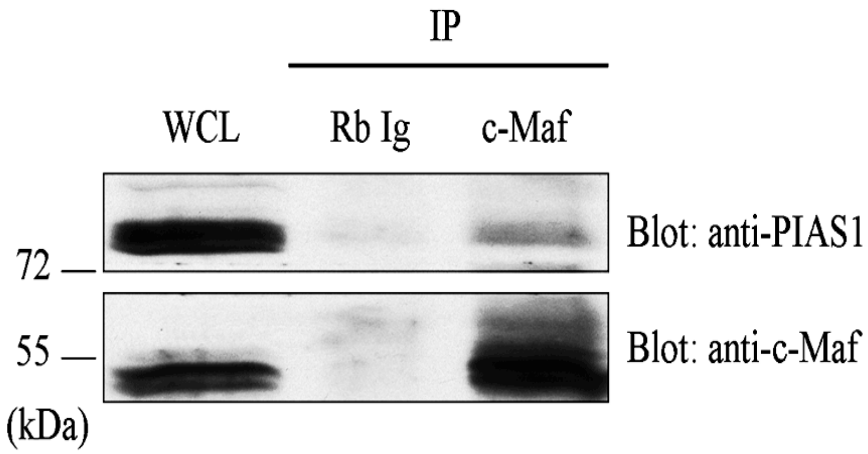

FIGURE 2. PIAS1 interacts with c-Maf in primary Th2 cells.

Naive $CD4^+$ T cells obtained from BALB/c mice were cultured under a Th2-skewing condition for four days. Cells were re-stimulated with 1 μg/ml of anti-CD3 antibody, and then lysed in NP-40 lysis buffer. The whole cell lysate (WCL) was immunoprecipitated with anti-c-Maf antibody or the control rabbit IgG (Rb Ig) and the immunoprecipitates were eluted and analyzed by Western blotting with anti-PIAS1 or anti-c-Maf antibodies.

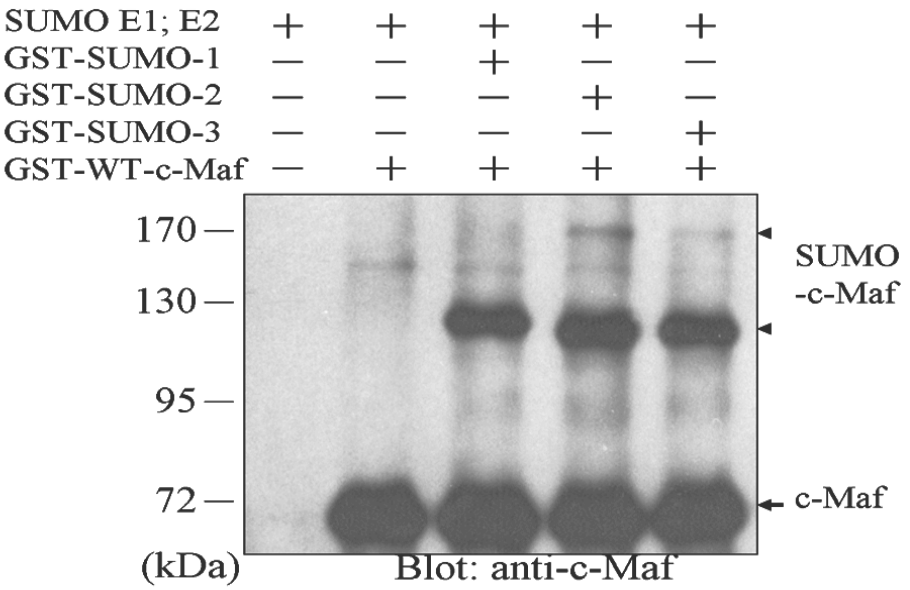

FIGURE 3. *In vitro* SUMOylation assay of c-Maf.

GST-tagged c-Maf was purified and incubated in the reaction mixture containing SUMO-E1, -E2, ATP and the indicated SUMOs at 30°C in water bath for 1 hour. The reaction mixtures were immunoblotted with anti-c-Maf antibody. The arrow and arrowhead indicate native and SUMO-modified c-Maf, respectively.

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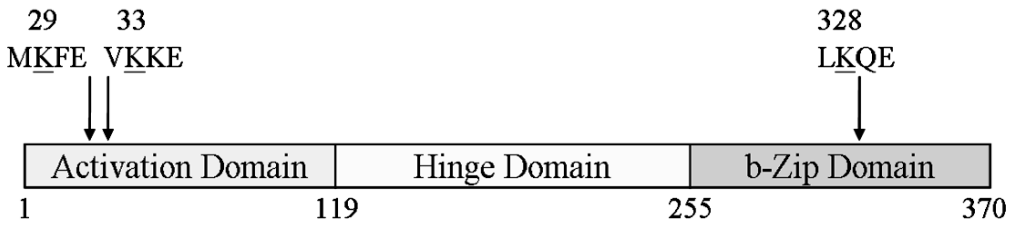
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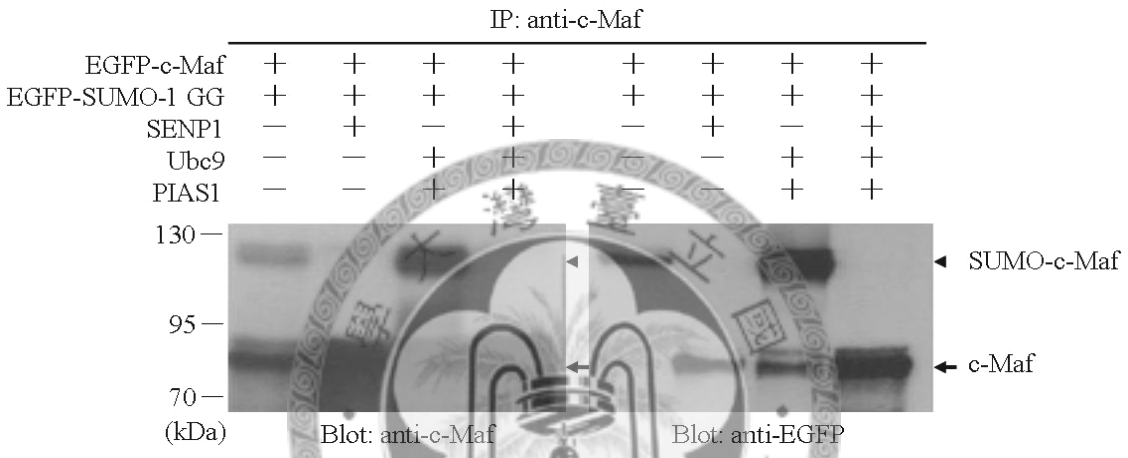
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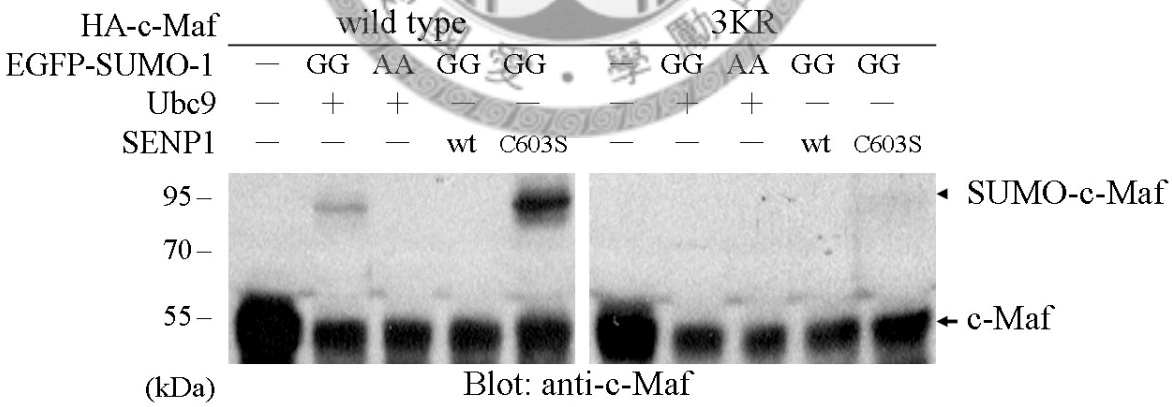
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c-Maf
Blot: anti-c-Maf

FIGURE 4. *In vitro* SUMOylation assay of c-Maf.

(A) The putative SUMO conjugating sites of c-Maf protein. The three predicted SUMO sites (ΨKXE) are indicated. The first two putative SUMO sites locate in the activation domain, and the third putative SUMO site is in the b-Zip domain. The lysine (K) for SUMO conjugation is underlined. The numbers represent the numbers of the amino acid residues. **(B) *In vivo* SUMOylation of c-Maf in RK13 cells.** 2×10^5 RK13 cells were transfected with the indicated combination of pEGFP-c-Maf, pEGFP-Sumo-1 GG, pFlag-Wt-SENP-1, pFlag- Wt-Ubc9 or pMyc-Wt- PIAS1. Twenty-four hours post-transfection, cells were lysed by lysis buffer and the cell lysates were immunopericipated with anti-c-Maf antibody. The immunoprecipitants were immunoblotted with anti-c-Maf (left) or anti-EGFP (right) antibodies for detection of c-Maf. **(C) *In vivo* SUMOylation of c-Maf in HEK293T cells.** HEK293T cells (2×10^5) were co-transfected with pcDNA3.1 HA-WT- or HA-K29,33,328R-c-Maf, cobined with the pFlag-Wt-Ubc9, pFlag-Wt-SENP1 or pFlag-C603S-SENP1. Cells were lysed and the cell lysates were immunoblotted with anti-c-Maf antibody. The arrow and arrowhead indicate native and SUMO-modified c-Maf, respectively.

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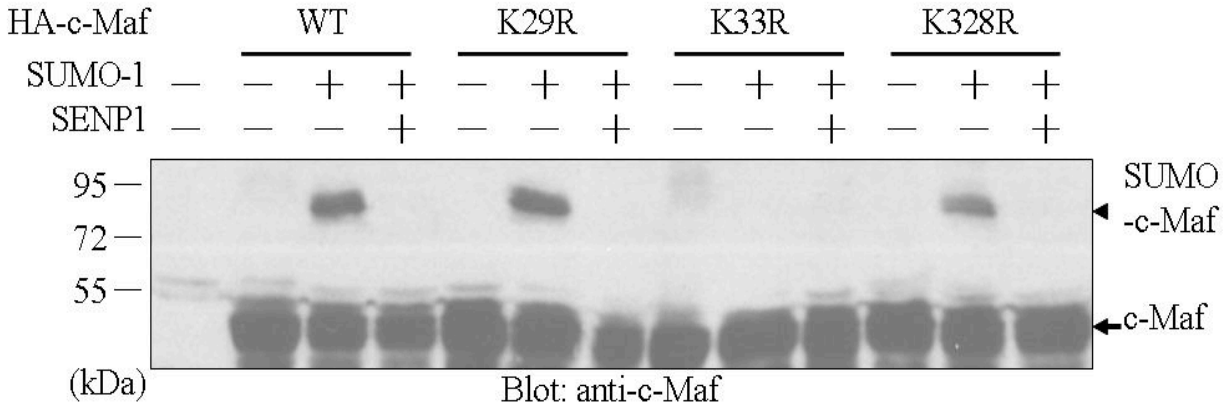

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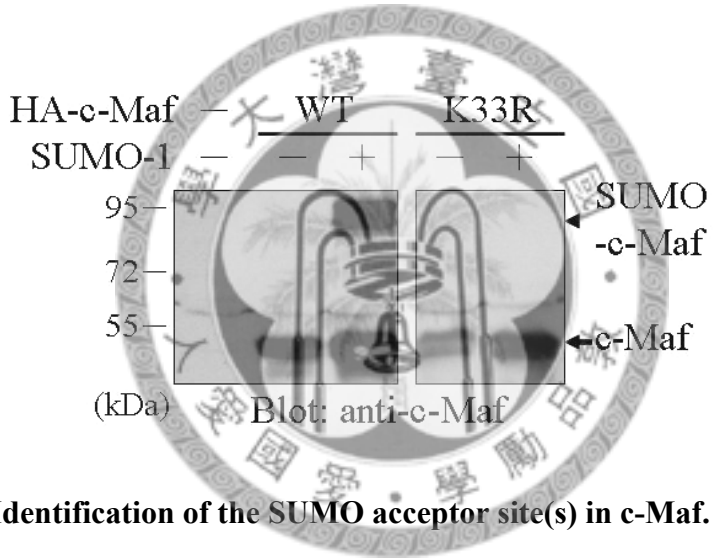
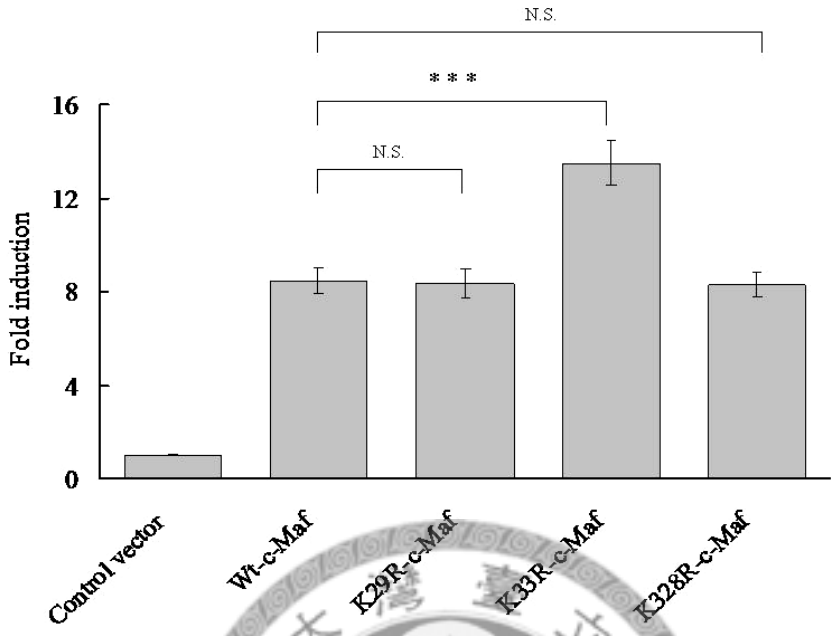


FIGURE 5. Identification of the SUMO acceptor site(s) in c-Maf.

(A) K33R is the SUMO accepter site of c-Maf. HEK293T cells (2×10^5) were transfected with pcDNA3.1 encoding HA-Wt-, HA-K29R-, HA-K33R- or HA-K328R-c-Maf, combined with the indicated expression vectors. Twenty four hours later, cells were lysed and immunoblotted with anti-c-Maf antibody. **(B) Wt-c-Maf, but not its K33R-c-Maf mutant, undergoes SUMOylation in DO11.10 T cell line.** DO11.10 cells (2×10^6) were transfected with pEGFP-WT- or -K33R-c-Maf, combined with or without EGFP-SUMO expression vector. Cell lysates were collected and immunoblotted with anti-c-Maf antibody. The arrow and arrowhead indicate native and SUMO-modified c-Maf, respectively.

A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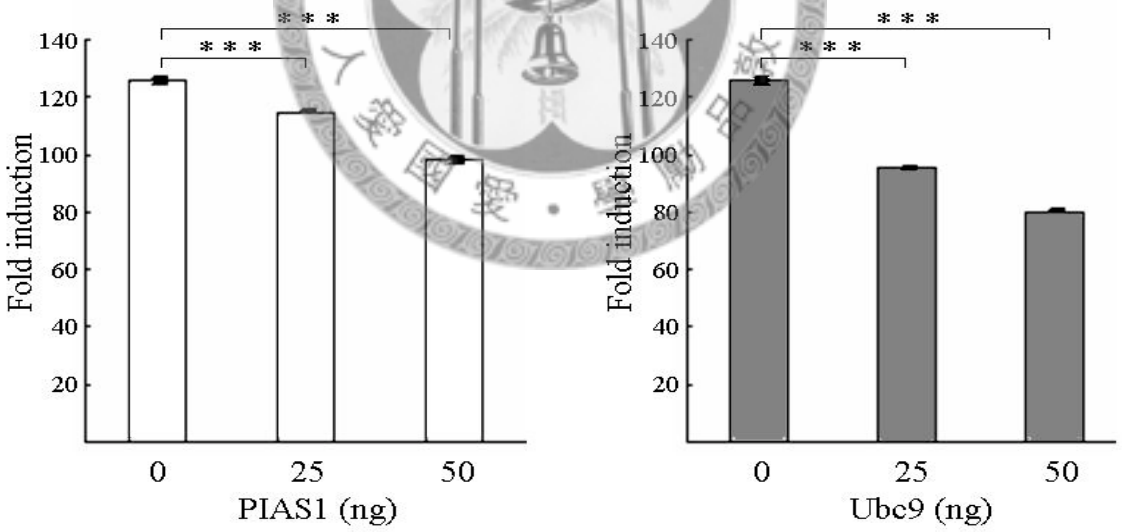
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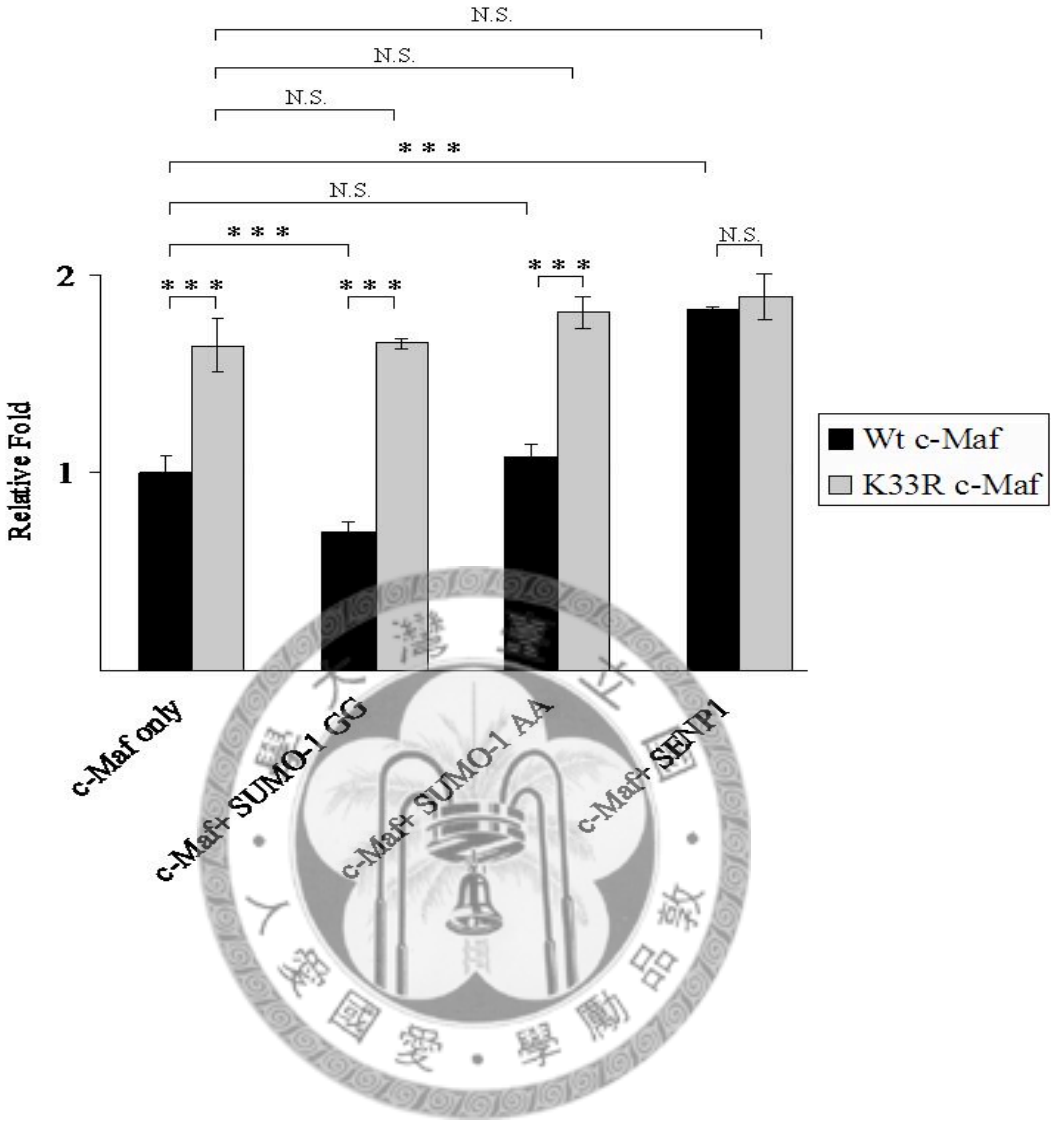
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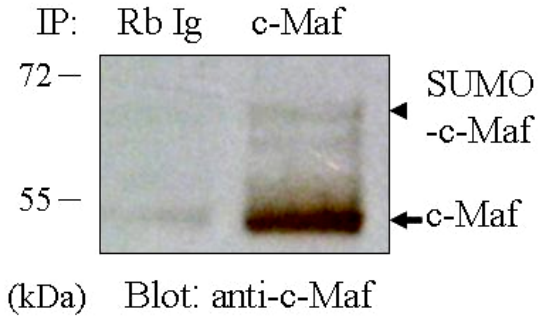
Relative Fold
Wt c-Maf
K33R c-Maf
c-Maf only
c-Maf+ SUMO-1 GG
c-Maf+ SUMO-1 AA
c-Maf+ SENP1

FIGURE 6. The transcription activity of c-Maf is suppressed by SUMOylation.

(A) SUMO-deficient c-Maf has higher MARE promoter transactivity. HEK293T cells were cotransfected with 2X MARE-promoter luciferase reporter together with Wt-, K29R-, K33R- or K328R-c-Maf expression vector. **(B) PIAS1 and UBC9 suppressed the c-maf dependent *Il4* promoter transactivity.** 2×10^5 293T cells were transfected with 50ng of *Il4* promoter-luciferase reporter vector and 25ng c-maf, combined with 0, 25, 50ng PIAS1 (left) or UBC9 (right), respectively. **(C) c-Maf transactivation ability were suppressed by SUMO modification.** HEK293T cells were cotransfected with luciferase reporter plasmids, driven by IL-4 (IL-4 Luc) promoters, together with a wild-type or K33R c-Maf expression vector plus wild-type or mutated Sumo-1 or Senp-1. Cells were lysed 24hrs later, and cell extracts were analyzed with the Dual-Glo Luciferase Assay System (Promega). All luciferase data were correlated by co-transfected Renilla expression level. The promoter-luciferase activity observed by transfection of the empty expression vector was normalized to 1. Mean and SD values (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.) were obtained from three independent samples (n=3).

A


IP: Rb Ig c-Maf
72
55
SUMO
-c-Maf
c-Maf
(kDa) Blot: anti-c-Maf

B

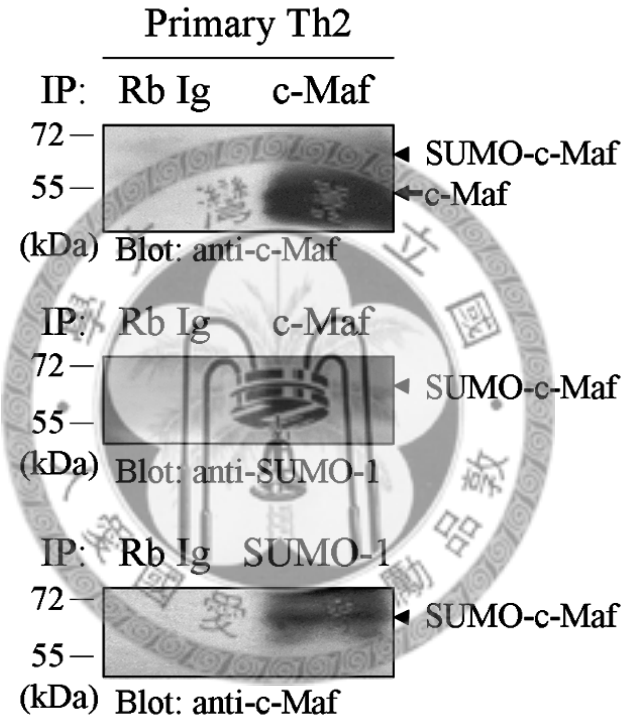
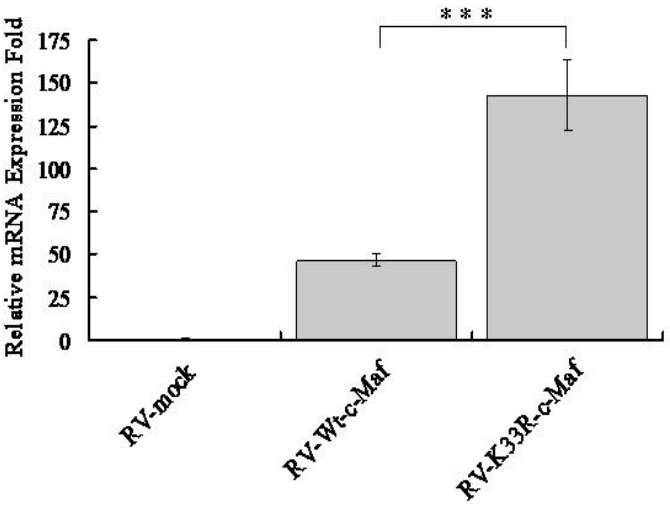

Primary Th2
IP: Rb Ig c-Maf
72
55
SUMO-c-Maf
c-Maf
(kDa) Blot: anti-c-Maf
IP: Rb Ig c-Maf
72
55
SUMO-c-Maf
(kDa) Blot: anti-SUMO-1
IP: Rb Ig SUMO-1
72
55
SUMO-c-Maf
(kDa) Blot: anti-c-Maf

FIGURE 7. c-Maf is SUMOylated in T cells.

(A) *In vivo* SUMOylation of c-Maf in the D10 Th2 clone. D10 cell lysates were collected after being stimulated for 4 hrs with PMA/ionomycin. The cell lysates were immunopericipated with anti-c-Maf antibody. The rabbit IgG (Rb Ig) was used as a control. The precipitants were washed five times and analyzed using Western blotting with anti-c-Maf antibody. **(B) *In vivo* SUMOylation of c-Maf in primary Th2 cells.** Primary cells were restimulated with anti-CD3 antibody for 24 hours before lysis by NP-40 lysis buffer containing 20 mM NEM. Cell lysate was immunoprecipitated with anti-c-Maf (top and middle) or anti-SUMO-1 (bottom) antibody. The rabbit IgG (Rb Ig) was used as a control. The precipitates were washed five times and subjected to immunoblotting with anti-SUMO-1 (middle) or anti-c-Maf (top and bottom) antibody. The arrows indicate the original non-SUMOylated-c-Maf and the arrowheads indicate the SUMO-modified-c-Maf.

A


* * *
Relative mRNA Expression Fold
175
150
125
100
75
50
25
0
RV-mock
RV-Wt-c-Maf
RV-K33R-c-Maf

B

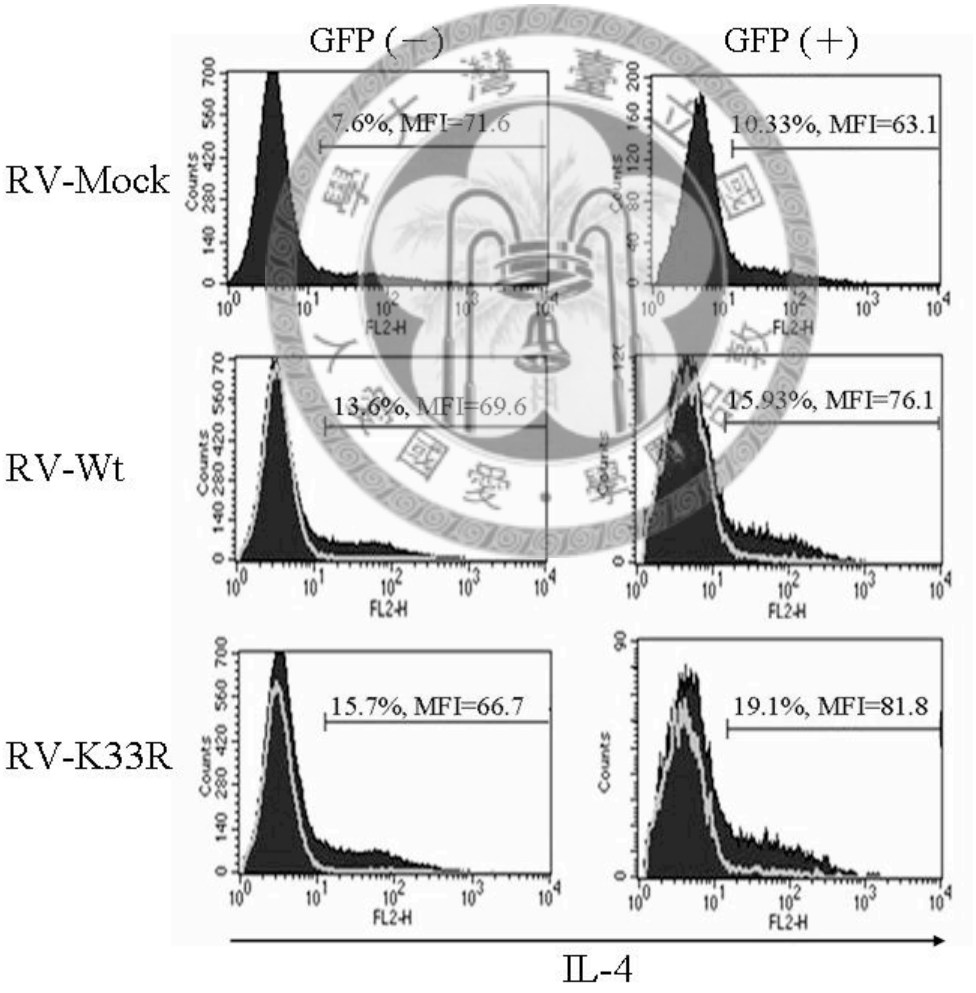
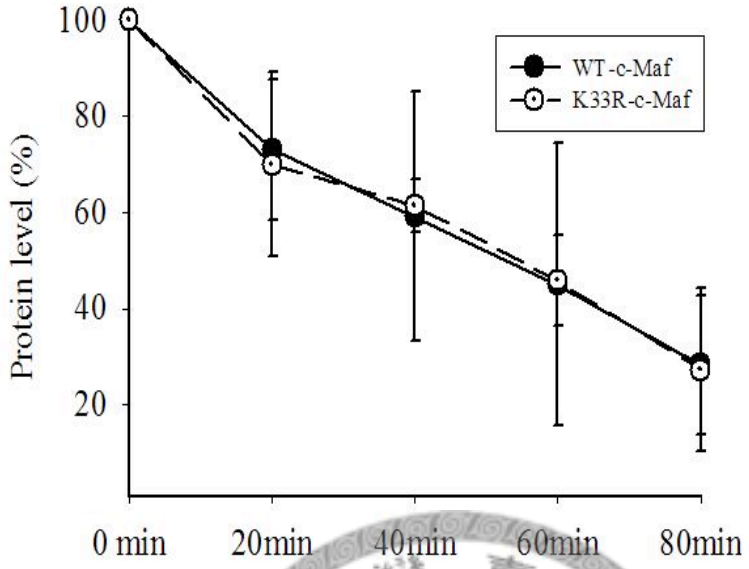

GFP (−)
GFP (+)
RV-Mock
RV-Wt
RV-K33R
7.6%, MFI=71.6
10.33%, MFI=63.1
13.6%, MFI=69.6
15.93%, MFI=76.1
15.7%, MFI=66.7
19.1%, MFI=81.8
Counts
FL2-H
IL-4

FIGURE 8. K33R-c-Maf induces more Il-4 than Wt-c-Maf.

(A) SUMO-site mutated c-Maf induced more IL-4 mRNA expression. Naïve CD4+ T cells isolated from BALB/c mice were activated by anti-CD3/anti-CD28 antibobies under Th0 skewing conditions and then transduced with wild-type c-Maf, K33R c-Maf or Mock GFP-RV. On the day 4, GFP+ cells were sorted and restimulated by PMA plus Ionomycin for 4 hours. The mRNA were extracted in order to perform the quantitative real time PCR. The relative expression fold was compared to the Mock retroviral transduced GFP+ cells as 1. Mean and SD values (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.) were obtained from three independent samples (n=3). **(B) SUMO-site mutated c-Maf induced more IL-4 producing helper T cells.** $CD4^+$ T cells isolated from lymph nodes were activated by anti-CD3 and anti-CD28 antibobies under Th0 conditions and then transduced with wild-type c-Maf, K33R c-Maf or Mock (GFP) retrovirus thirty hours later. Cells were cultured for another three days and restimulated by PMA plus Ionomycin for 4 hours in order to performed the intracellular cytokine staining assay. The percentages indicate the IL-4 producing cells. GFP (+) mean the retrovirus transduced cells, and GFP (-) are untransduced cells.

A


100
80
60
40
20
Protein level (%)
WT-c-Maf
K33R-c-Maf
0 min
20min
40min
60min
80min
Time

B

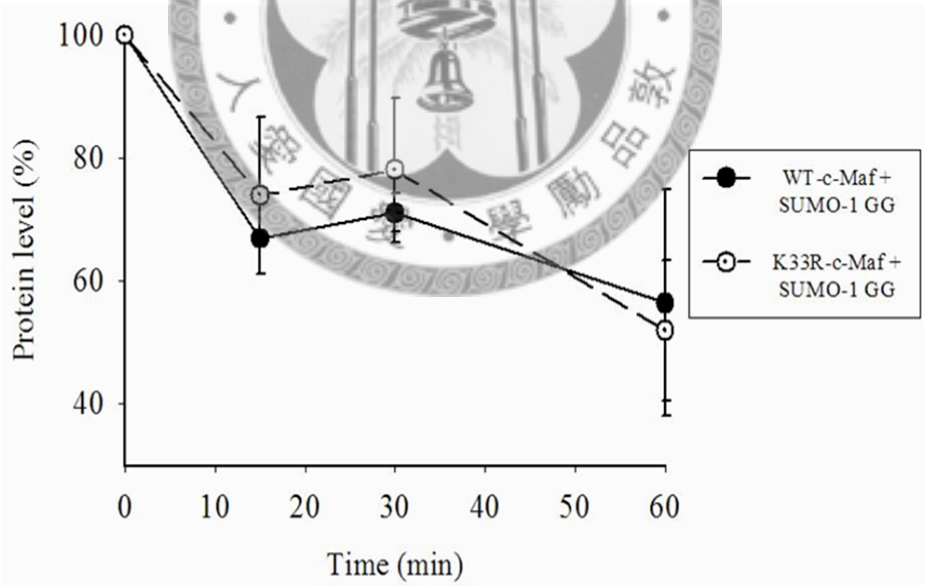
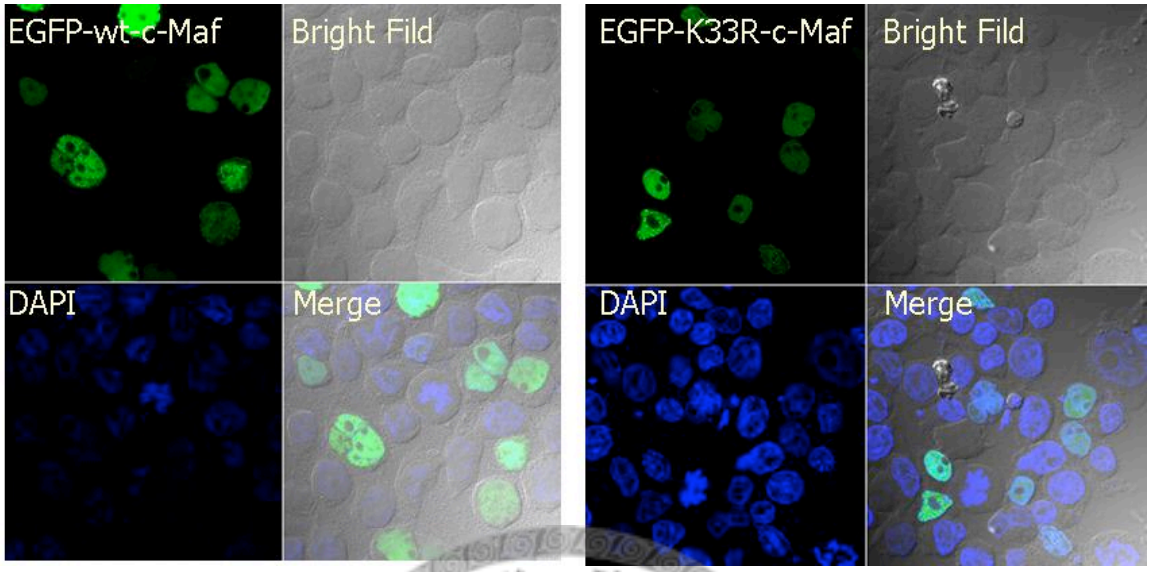

100
80
60
40
Protein level (%)
WT-c-Maf+
SUMO-1 GG
K33R-c-Maf+
SUMO-1 GG
0
10
20
30
40
50
60
Time (min)

FIGURE 9. SUMOylation does not affect c-Maf protein stability.

(A) Wt- and SUMO deficient K33R-c-Maf have the same helf-lives. 3×10^6 HEK293T cells were transfected with HA tagged wild-type or K33R-c-Maf. One day after transfection, cells were resuspended in 6-well culture plates and treated with 20 μg/ml cycloheximide. Cells were collected for different time periods and then immunoblotted with anti-c-Maf antibody. The relative protein levels are shown on the figures. **(B) SUMOylation does not affect c-Maf protein stability.** HEK293T cells (3×10^6) were co-transfected with EGFP-SUMO-1-GG and a vector expressing HA-tagged WT- or K33R-c-Maf. The stability of c-Maf protein were measured as described in materials and methods. c-Maf protein level was normalized against actin level of the same sample and compared to the normalized c-Maf level at time 0, which was arbitrarily set as 1. The mean and SD values of data collected from three independent experiments are shown.

A


EGFP-wt-c-Maf
Bright Fild
DAPI
Merge
EGFP-K33R-c-Maf
Bright Fild
DAPI
Merge

B

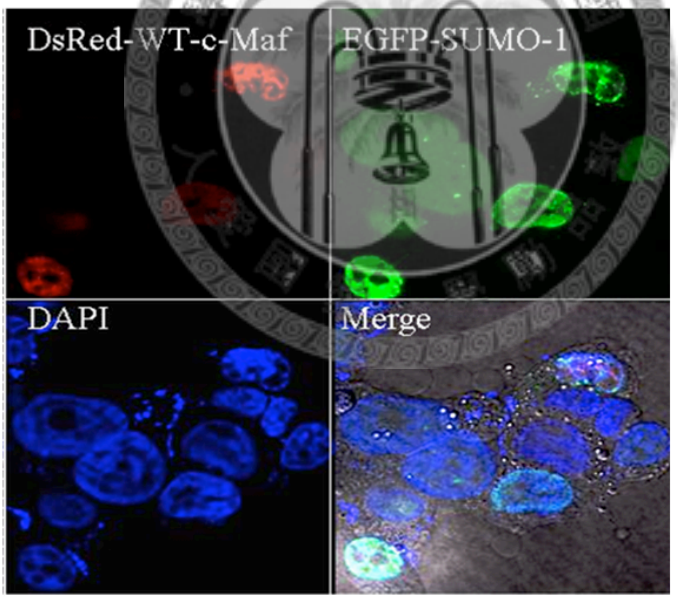

DsRed-WT-c-Maf
EGFP-SUMO-1
DAPI
Merge

FIGURE 10. Localization of Wt- and K33R-c-Maf are not affect by SUMO.

(A) The cellular localization of WT- and K33R-c-Maf. HEK 293T cells (4×10^5) were transfected with a vector expressing EGFP-WT- or -K33R-c-Maf respectively. Cells were fixed and stained with nucleus indicator DAPI (blue), and the localization of c-Maf was viewed under a confocal microscope. **(B) SUMOylation does not affect the cellular localization of WT-c-Maf.** HEK 293T cells (4×10^5) were transfected with vectors expressing DsRed-c-Maf and EGFP-SUMO-1. Cells were fixed and stained with nucleus indicator DAPI (blue). The localization of the c-Maf and SUMO-1 were viewed under a confocal microscope.

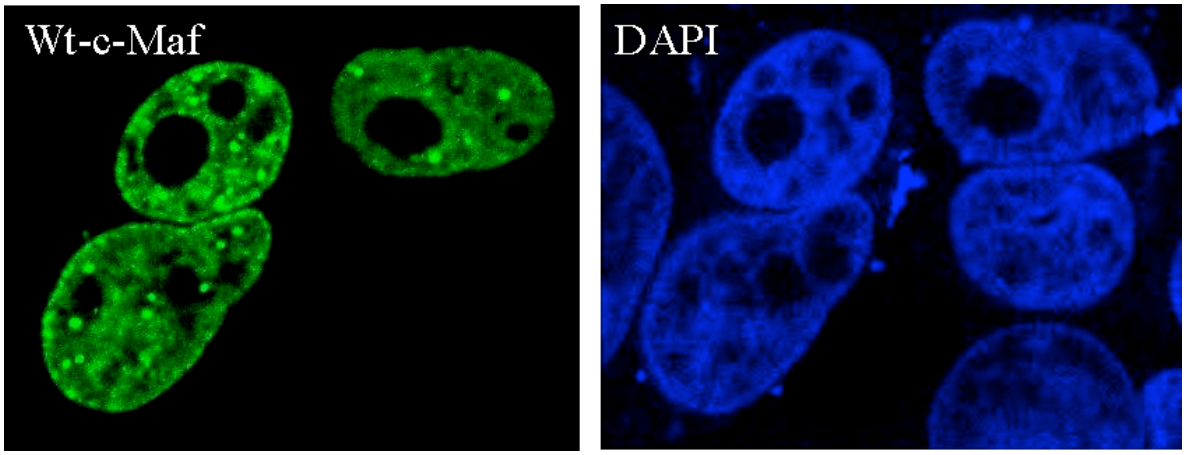
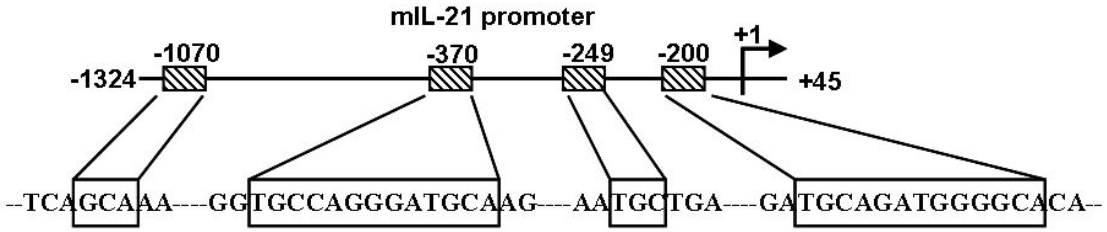


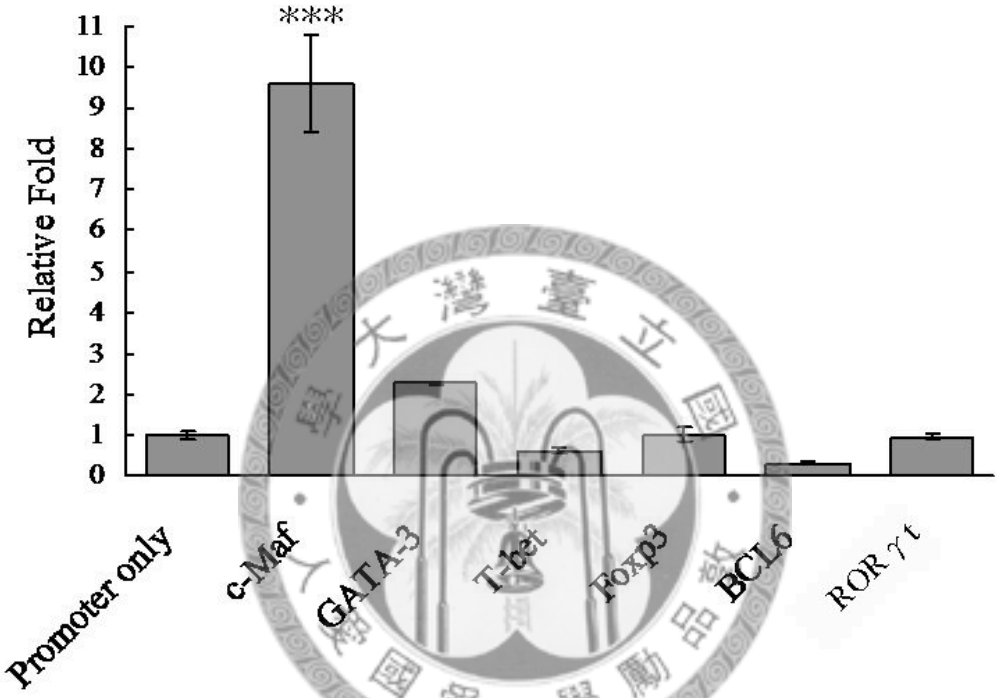
FIGURE 11. Wt-c-Maf concentrates within in the nuclear spot

HEK 293T cells (4×10^5) were transfected with a vector expressing EGFP-Wt-c-Maf for one day. Cells were fixed and stained with DAPI (blue), and the localization of c-Maf was viewed under a confocal microscope.

A


mIL-21 promoter
-1324
-1070
-370
-249
-200
+1
+45
--TCAGCAAA----GGTGCCAGGGATGCAAG----AATGCTGA----GATGCAGATGGGGCACA--

B


***
Relative Fold
11
10
9
8
7
6
5
4
3
2
1
0
Promoter only
c-Maf
GATA-3
T-bet
Foxp3
BCL6
RORγt

C

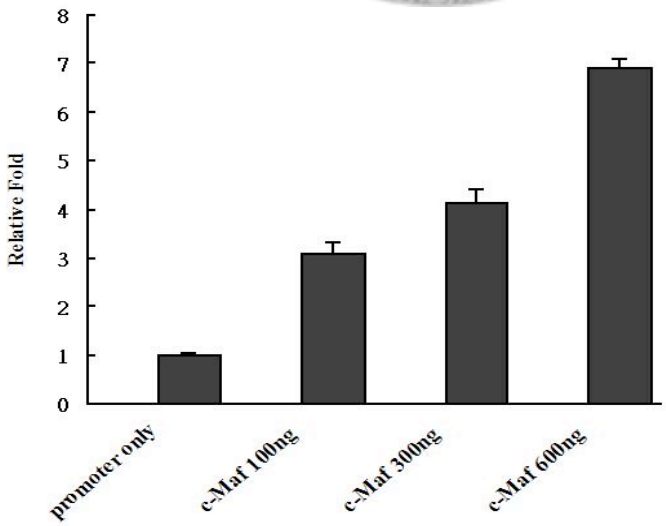

Relative Fold
8
7
6
5
4
3
2
1
0
promoter only
c-Maf 100ng
c-Maf 300ng
c-Maf 600ng

FIGURE 12. c-Maf is the specific transcription factor for IL-21 gene expression.

(A) Schematic representation of the proximal regulatory region and the putative MARE sites on the murine IL-21 promoter. The figure shows the primary sequence of the murine IL-21 promoter. The numbers indicated are relative to the start site of transcriptionat +1. **(B) c-Maf specifically transactivates IL-21 promoter-repoter gene expression.** HEK293 T cells were cotransfection of the IL-21-luciferase reporter plasmid (300 ng), combined with the control empty vector (900 ng), the c-Maf (900 ng), GATA-3 (900 ng), T-bet (900 ng), Foxp3 (900 ng) BCL6 (900 ng) or RORγt vector (900 ng). Promoter activity was quantified by a luciferase assay 24 h post-transfection. **(C) c-Maf transactivates the IL-21 promoter-reporter in dose-dependent manner.** HEK293T cells were cotransfected with 300 ng IL-21-luciferase reporter plasmid plus three different concentrations of c-Maf expression vector (100, 300 or 600 ng) for 24 h, and the promoter activity was quantified. The promoter-luciferase activity observed by transfection of the empty expression vector was normalized to 1. Mean and SD values (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.) were obtained from three independent samples (n=3). Each experiment was performed at least three times with similar results.

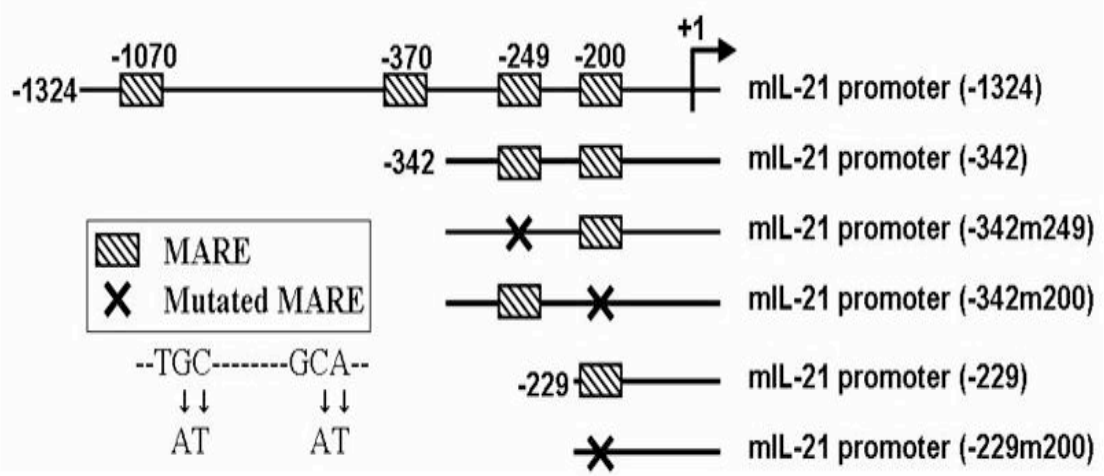
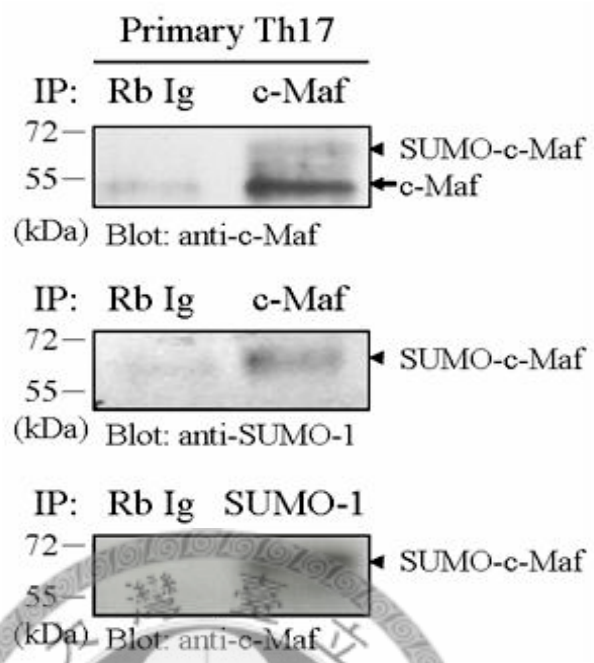


FIGURE 13. c-Maf transcativates IL-21 through the IL-21 promoter proximal MARE site. Schematic representation of the different constructs of the IL-21 promoter-repoter vector. The mutant MARE was generated by replacing the conserve MARE sequence TGC and GCA to TAT and GAT. The numbers indicated are relative to the start site of transcriptionat +1.

A


Primary Th17
IP: Rb Ig c-Maf
72—
55—
SUMO-c-Maf
c-Maf
(kDa) Blot: anti-c-Maf
IP: Rb Ig c-Maf
72—
55—
SUMO-c-Maf
(kDa) Blot: anti-SUMO-1
IP: Rb Ig SUMO-1
72—
55—
SUMO-c-Maf
(kDa) Blot: anti-c-Maf

B

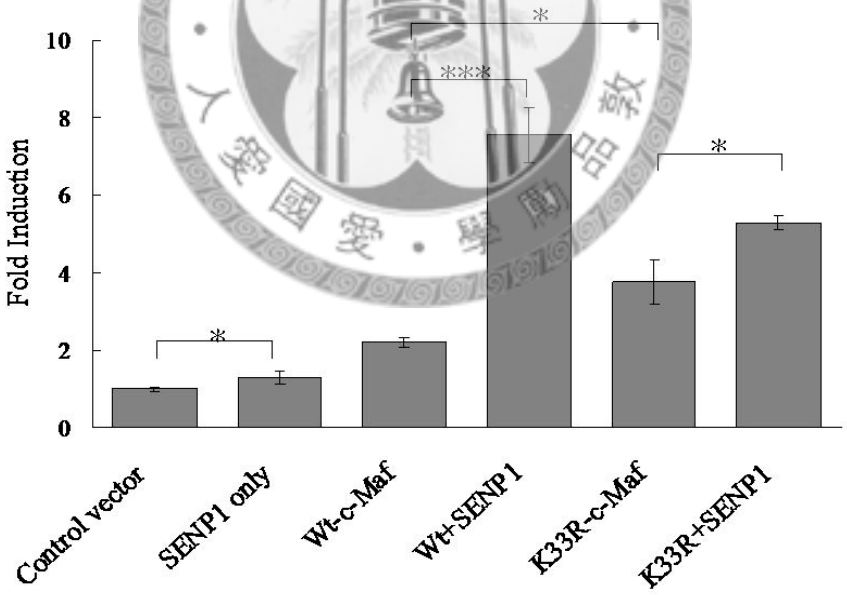

Fold Induction
10
8
6
4
2
0
*
***
*
*
Control vector
SENP1 only
Wt-c-Maf
Wt+SENP1
K33R-c-Maf
K33R+SENP1

FIGURE 14. c-Maf is SUMOylated inTh17 cells and attenuates its IL-21 transactivity.

(A) *In vivo* SUMOylation of c-Maf in primary Th17 cells. Primary Th17 cells were restimulated with anti-CD3 antibody for 24 hours before lysis by NP-40 lysis buffer containing 20 mM NEM. Cell lysate was immunoprecipitated with anti-c-Maf (top and middle) or anti-SUMO-1 (bottom) antibody. The rabbit IgG (Rb Ig) was used as a control. The precipitates were washed five times and subjected to immunoblotting with anti-SUMO-1 (middle) or anti-c-Maf (top and bottom) antibody. The arrows indicate the original non-SUMOylated-c-Maf and the arrowheads indicate the SUMO-modified-c-Maf. **(B) SUMO deficient K33R c-Maf is more potent to induce the IL-21 gene expression.** HEK293T cells (2×10^5) were transfected with Renilla, *il21-promoter* luciferase reporter plasmid, and indicated expression vectors. Cell lysate was analyzed with the Dual-Glo Luciferase Assay System (Promega). *Il21-promoter*-luciferase activity was normalized against renilla luciferase and compared with the normalized activity of the empty control vector, which was arbitrarily set at 1. Mean and SD values (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.) were obtained from three independent samples (n=3).

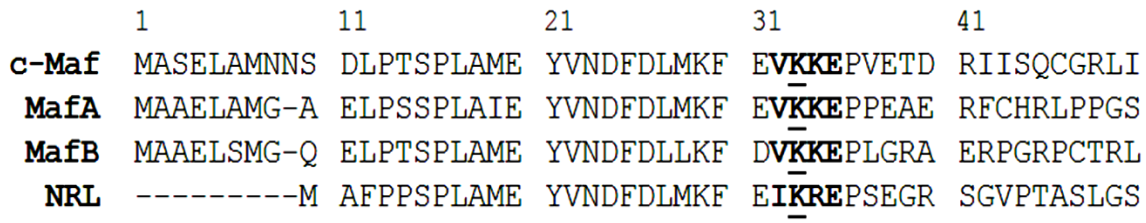


FIGURE 15. Protein alignment of SUMO-conjugating sites at the N-terminal of murine large Maf family. K33, K32, K32 and K24 (bold and underlined) are the conserved lysine residue in the AD (bold) of c-Maf, MafA, MafB and NRL respectively. Note that K24 of NRL is a putative SUMO acceptor site. The protein sequence is aligned to K33 of c-Maf.

TABLE 1. c-Maf interacting protein candidates list.

The yeast two-hybrid assay screened about 2×10^6 colonies. After checked by nutrient selection plate and β-gal colony-lift filter assay, 954 colonies were selected. The cDNA library carring vectors in these colonies were extracted and then grouped according to the inserted cDNA size and Alu I digestion patterns in order to exclude sister clones. Finally, 23 groups of candidate were found and sequenced. The SUMO E2 and E3 ligase, Ubc9 (group 1) and the PIAS1 (group 6), were isolated thirty and two times, respectively. The order of the list is arranged by the frequency.

Group	frequency	
1	**30**	**UBC9**
2	11	Reversed insert
3	3	Ribosomal RNA protein
4	3	snRNP70
5	2	Cadherin-related neuronal receptor
6	**2**	**PIAS1**
7	1	Actin related protein 2/3 complex subunit 2
8	1	Cytokine like nuclear factor n-pac like protein
9	1	GM-CSF (not in frame)
10	1	Gp94A(B) (not in frame)
11	1	Guenine nucleotide binding protein like 2
12	1	Kif21B (not in frame)
13	1	mBC043098
14	1	mRP23212N20, chromosome5
15	1	mRP23-392213, chromosome11
16	1	mRP23-392I3, chromosome11
17	1	mRP24-308L17, chromosome 9
18	1	mRP24-571B18
19	1	Not in frame
20	1	pJG4-5 backbone
21	1	PolyA polymerase (like)
22	1	Receptor tyrosine kinase (untranslated region)
23	1	Synectin

References:

Bauquet, A.T., Jin, H., Paterson, A.M., Mitsdoerffer, M., Ho, I.C., Sharpe, A.H., and Kuchroo, V.K. (2009). The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. Nature immunology *10*, 167-175.

Bayer, P., Arndt, A., Metzger, S., Mahajan, R., Melchior, F., Jaenicke, R., and Becker, J. (1998). Structure determination of the small ubiquitin-related modifier SUMO-1. J Mol Biol *280*, 275-286.

Betz, B.C., Jordan-Williams, K.L., Wang, C., Kang, S.G., Liao, J., Logan, M.R., Kim, C.H., and Taparowsky, E.J. (2010). Batf coordinates multiple aspects of B and T cell function required for normal antibody responses. The Journal of experimental medicine *207*, 933-942.

Blank, V., and Andrews, N.C. (1997). The Maf transcription factors: regulators of differentiation. Trends Biochem Sci *22*, 437-441.

Bohren, K.M., Nadkarni, V., Song, J.H., Gabbay, K.H., and Owerbach, D. (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.

Cao, S., Liu, J., Chesi, M., Bergsagel, P.L., Ho, I.C., Donnelly, R.P., and Ma, X. (2002).

Differential regulation of IL-12 and IL-10 gene expression in macrophages by the basic leucine zipper transcription factor c-Maf fibrosarcoma. J Immunol *169*, 5715-5725.

Chen, D.Y., Chen, Y.M., Chen, H.H., Hsieh, C.W., Lin, C.C., and Lan, J.L. (2005). The associations of circulating CD4(+)CD25(high) regulatory T cells and TGF-beta with disease activity and clinical course in patients with adult-onset Still's disease. Connective tissue research.

Chen, Q., Yang, W., Gupta, S., Biswas, P., Smith, P., Bhagat, G., and Pernis, A.B. (2008). IRF-4-binding protein inhibits interleukin-17 and interleukin-21 production by controlling the activity of IRF-4 transcription factor. Immunity *29*, 899-911.

Dohmen, R.J. (2004). SUMO protein modification. Biochim Biophys Acta *1695*, 113-131.

Fantini, M.C., Rizzo, A., Fina, D., Caruso, R., Becker, C., Neurath, M.F., Macdonald, T.T., Pallone, F., and Monteleone, G. (2007). IL-21 regulates experimental colitis by modulating the balance between Treg and Th17 cells. European journal of immunology *37*, 3155-3163.

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nature immunology *4*, 330-336.

Fu, S., Zhang, N., Yopp, A.C., Chen, D., Mao, M., Chen, D., Zhang, H., Ding, Y., and

Bromberg, J.S. (2004). TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 - precursors. Am J Transplant *4*, 1614-1627.

Fujiwara, K.T., Kataoka, K., and Nishizawa, M. (1993). Two new members of the maf oncogene family, mafK and mafF, encode nuclear b-Zip proteins lacking putative trans-activator domain. Oncogene *8*, 2371-2380.

Gill, G. (2004). SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? Genes Dev *18*, 2046-2059.

Harada, M., Magara-Koyanagi, K., Watarai, H., Nagata, Y., Ishii, Y., Kojo, S., Horiguchi, S., Okamoto, Y., Nakayama, T., Suzuki, N., *et al.* (2006). IL-21-induced Bepsilon cell apoptosis mediated by natural killer T cells suppresses IgE responses. The Journal of experimental medicine *203*, 2929-2937.

Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M., and Weaver, C.T. (2005). Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nature immunology *6*, 1123-1132.

Hiramatsu, Y., Suto, A., Kashiwakuma, D., Kanari, H., Kagami, S., Ikeda, K., Hirose, K., Watanabe, N., Grusby, M.J., Iwamoto, I., and Nakajima, H. (2010). c-Maf activates the promoter and enhancer of the IL-21 gene, and TGF-beta inhibits c-Maf-induced IL-21 production in CD4+ T cells. Journal of leukocyte biology *87*, 703-712.

Ho, I., Vorhees, P., Marin, N., Oakley, B., Tsai, S., Orkin, S., and Leiden, J. (1991). Human GATA-3: a lineage-restricted transcription factor that regulates the expression of the T cell receptor alpha gene. EMBO J *10*, 1187-1192.

Ho, I.C., Hodge, M.R., Rooney, J.W., and Glimcher, L.H. (1996). The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. Cell *85*, 973-983.

Hodge, M.R., Chun, H.J., Rengarajan, J., Alt, A., Lieberson, R., and Glimcher, L.H. (1996). NF-AT-Driven interleukin-4 transcription potentiated by NIP45. Science *274*, 1903-1905.

Ivanov, II, McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., and Littman, D.R. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell *126*, 1121-1133.

Johnson, E.S. (2004). Protein modification by SUMO. Annu Rev Biochem *73*, 355-382.

Kataoka, K., Fujiwara, K.T., Noda, M., and Nishizawa, M. (1994a). MafB, a new Maf family transcription activator that can associate with Maf and Fos but not with Jun. Mol Cell Biol *14*, 7581-7591.

Kataoka, K., Igarashi, K., Itoh, K., Fujiwara, K.T., Noda, M., Yamamoto, M., and Nishizawa, M. (1995). Small Maf proteins heterodimerize with Fos and may act as competitive repressors of the NF-E2 transcription factor. Mol Cell Biol *15*, 2180-2190.

Kataoka, K., Nishizawa, M., and Kawai, S. (1993). Structure-function analysis of the maf oncogene product, a member of the b-Zip protein family. J Virol *67*, 2133-2141.

Kataoka, K., Noda, M., and Nishizawa, M. (1994b). Maf nuclear oncoprotein recognizes sequences related to an AP-1 site and forms heterodimers with both Fos and Jun. Molecular and cellular biology *14*, 700-712.

Kerppola, T.K., and Curran, T. (1994). A conserved region adjacent to the basic domain is required for recognition of an extended DNA binding site by Maf/Nrl family proteins. Oncogene *9*, 3149-3158.

Killar, L., MacDonald, G., West, J., Woods, A., and Bottomly, K. (1987). Cloned, Ia-restricted T cells that do not produce interleukin 4(IL 4)/B cell stimulatory factor 1(BSF-1) fail to help antigen-specific B cells. J Immunol *138*, 1674-1679.

Kim, H.P., Korn, L.L., Gamero, A.M., and Leonard, W.J. (2005). Calcium-dependent activation of interleukin-21 gene expression in T cells. The Journal of biological chemistry *280*, 25291-25297.

Kim, J.I., Ho, I.C., Grusby, M.J., and Glimcher, L.H. (1999). The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. Immunity *10*, 745-751.

King, C., Tangye, S.G., and Mackay, C.R. (2008). T follicular helper (TFH) cells in normal and dysregulated immune responses. Annual review of immunology *26*,

741-766.

Ko, L.J., Yamamoto, M., Leonard, M.W., George, K.M., Ting, P., and Engel, J.D. (1991). Murine and human T-lymphocyte GATA-3 factors mediate transcription through a cis-regulatory element within the human T-cell receptor delta gene enhancer. Mol Cell Biol *11*, 2778-2784.

Korn, T., Bettelli, E., Gao, W., Awasthi, A., Jager, A., Strom, T.B., Oukka, M., and Kuchroo, V.K. (2007). IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature *448*, 484-487.

Leavenworth, J.W., Ma, X., Mo, Y.Y., and Pauza, M.E. (2009). SUMO Conjugation Contributes to Immune Deviation in Nonobese Diabetic Mice by Suppressing c-Maf Transactivation of IL-4. J Immunol.

Lee, H., Takemoto, N., Kurata, H., Kamogawa, Y., Miyatake, S., O'Garra, A., and Arai, N. (2000). GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. J Exp Med *192*, 105-115.

Lighvani, A., Frucht, D., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B., Nguyen, B., Gadina, M., Sher, A., Paul, W., and O'Shea, J. (2001). T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. Proc Natl Acad Sci U S A *98*, 15137-15142.

Lin, B.S., Tsai, P.Y., Hsieh, W.Y., Tsao, H.W., Liu, M.W., Grenningloh, R., Wang, L.F.,

Ho, I.C., and Miaw, S.C. (2010) SUMOylation attenuates c-Maf-dependent IL-4 expression. European journal of immunology. 40, 1174-1184.

Mehta, D.S., Wurster, A.L., Weinmann, A.S., and Grusby, M.J. (2005). NFATc2 and T-bet contribute to T-helper-cell-subset-specific regulation of IL-21 expression. Proceedings of the National Academy of Sciences of the United States of America *102*, 2016-2021.

Melchior, F. (2000). SUMO--nonclassical ubiquitin. Annu Rev Cell Dev Biol *16*, 591-626.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., and Coffman, R.L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol *136*, 2348-2357.

Mullen, A.C., Hutchins, A.S., High, F.A., Lee, H.W., Sykes, K.J., Chodosh, L.A., and Reiner, S.L. (2002). Hlx is induced by and genetically interacts with T-bet to promote heritable T(H)1 gene induction. Nature immunology *3*, 652-658.

Muller, S., Hoege, C., Pyrowolakis, G., and Jentsch, S. (2001). SUMO, ubiquitin's mysterious cousin. Nat Rev Mol Cell Biol *2*, 202-210.

Muller, S., Ledl, A., and Schmidt, D. (2004). SUMO: a regulator of gene expression and genome integrity. Oncogene *23*, 1998-2008.

Nishizawa, M., Kataoka, K., Goto, N., Fujiwara, K.T., and Kawai, S. (1989). v-maf, a viral oncogene that encodes a "leucine zipper" motif. Proc Natl Acad Sci U S A *86*, 7711-7715.

Nurieva, R., Yang, X.O., Martinez, G., Zhang, Y., Panopoulos, A.D., Ma, L., Schluns, K., Tian, Q., Watowich, S.S., Jetten, A.M., and Dong, C. (2007). Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature *448*, 480-483.

Ogino, H., and Yasuda, K. (1998). Induction of lens differentiation by activation of a bZIP transcription factor, L-Maf. Science *280*, 115-118.

Ohbayashi, N., Kawakami, S., Muromoto, R., Togi, S., Ikeda, O., Kamitani, S., Sekine, Y., Honjoh, T., and Matsuda, T. (2008). The IL-6 family of cytokines modulates STAT3 activation by desumoylation of PML through SENP1 induction. Biochemical and biophysical research communications *371*, 823-828.

Ouyang, W., Lohning, M., Gao, Z., Assenmacher, M., Ranganath, S., Radbruch, A., and Murphy, K. (2000). Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. Immunity *12*, 27-37.

Pandolfi, P.P., Roth, M.E., Karis, A., Leonard, M.W., Dzierzak, E., Grosveld, F.G., Engel, J.D., and Lindenbaum, M.H. (1995). Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. Nat Genet *11*, 40-44.

Park, H., Li, Z., Yang, X.O., Chang, S.H., Nurieva, R., Wang, Y.H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., and Dong, C. (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nature immunology *6*, 1133-1141.

Pot, C., Jin, H., Awasthi, A., Liu, S.M., Lai, C.Y., Madan, R., Sharpe, A.H., Karp, C.L., Miaw, S.C., Ho, I.C., and Kuchroo, V.K. (2009). Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. J Immunol *183*, 797-801.

Rengarajan, J., Mowen, K.A., McBride, K.D., Smith, E.D., Singh, H., and Glimcher, L.H. (2002). Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. The Journal of experimental medicine *195*, 1003-1012.

Saitoh, H., and Hinchey, J. (2000). Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. J Biol Chem *275*, 6252-6258.

Saitoh, H., Pu, R.T., and Dasso, M. (1997). SUMO-1: wrestling with a new ubiquitin-related modifier. Trends Biochem Sci *22*, 374-376.

Schraml, B.U., Hildner, K., Ise, W., Lee, W.L., Smith, W.A., Solomon, B., Sahota, G., Sim, J., Mukasa, R., Cemerski, S.*, et al.* (2009). The AP-1 transcription factor Batf controls T(H)17 differentiation. Nature *460*, 405-409.

Seeler, J.S., and Dejean, A. (2003). Nuclear and unclear functions of SUMO. Nat Rev

Mol Cell Biol *4*, 690-699.

Shao, C., and Cobb, M.H. (2009). Sumoylation regulates the transcriptional activity of MafA in pancreatic beta cells. The Journal of biological chemistry *284*, 3117-3124.

Sherman, M.A., Nachman, T.Y., and Brown, M.A. (1999). Cutting edge: IL-4 production by mast cells does not require c-maf. J Immunol *163*, 1733-1736.

Szabo, S., Kim, S., Costa, G., Zhang, X., Fathman, C., and Glimcher, L. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell *100*, 655-669.

Szabo, S., Sullivan, B., Stemmann, C., Satoskar, A., Sleckman, B., and Glimcher, L. (2002). Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. Science *295*, 338-342.

Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G., and Glimcher, L.H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell *100*, 655-669.

Szabo, S.J., Sullivan, B.M., Stemmann, C., Satoskar, A.R., Sleckman, B.P., and Glimcher, L.H. (2002). Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. Science (New York, N.Y *295*, 338-342.

Tatham, M.H., Jaffray, E., Vaughan, O.A., Desterro, J.M., Botting, C.H., Naismith, J.H.,

and Hay, R.T. (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.

Tillmanns, S., Otto, C., Jaffray, E., Du Roure, C., Bakri, Y., Vanhille, L., Sarrazin, S., Hay, R.T., and Sieweke, M.H. (2007). SUMO modification regulates MafB-driven macrophage differentiation by enabling Myb-dependent transcriptional repression. Molecular and cellular biology *27*, 5554-5564.

Ting, C.N., Olson, M.C., Barton, K.P., and Leiden, J.M. (1996). Transcription factor GATA-3 is required for development of the T-cell lineage. Nature *384*, 474-478.

Usui, T., Nishikomori, R., Kitani, A., and Strober, W. (2003). GATA-3 Suppresses Th1 Development by Downregulation of Stat4 and Not through Effects on IL-12Rbeta2 Chain or T-bet. Immunity *18*, 415-428.

Veldhoen, M., Uyttenhove, C., van Snick, J., Helmby, H., Westendorf, A., Buer, J., Martin, B., Wilhelm, C., and Stockinger, B. (2008). Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. Nature immunology *9*, 1341-1346.

Wan, Y.Y., and Flavell, R.A. (2007). Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature *445*, 766-770.

Wurster, A.L., Rodgers, V.L., Satoskar, A.R., Whitters, M.J., Young, D.A., Collins, M., and Grusby, M.J. (2002). Interleukin 21 is a T helper (Th) cell 2 cytokine that

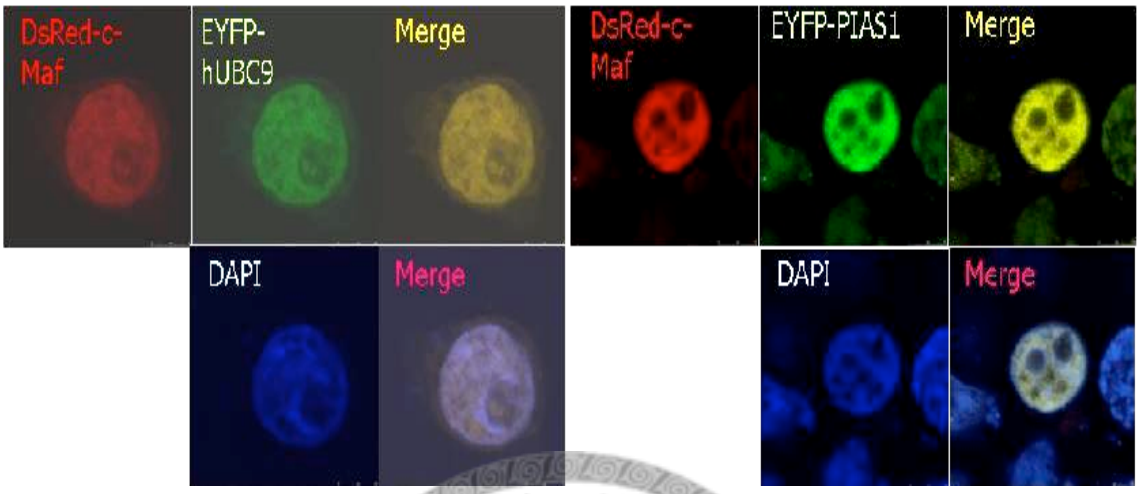
specifically inhibits the differentiation of naive Th cells into interferon gamma-producing Th1 cells. The Journal of experimental medicine *196*, 969-977.

Yang, X.O., Pappu, B.P., Nurieva, R., Akimzhanov, A., Kang, H.S., Chung, Y., Ma, L., Shah, B., Panopoulos, A.D., Schluns, K.S., *et al.* (2008). T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity *28*, 29-39.

Zhao, J. (2007). Sumoylation regulates diverse biological processes. Cell Mol Life Sci *64*, 3017-3033.

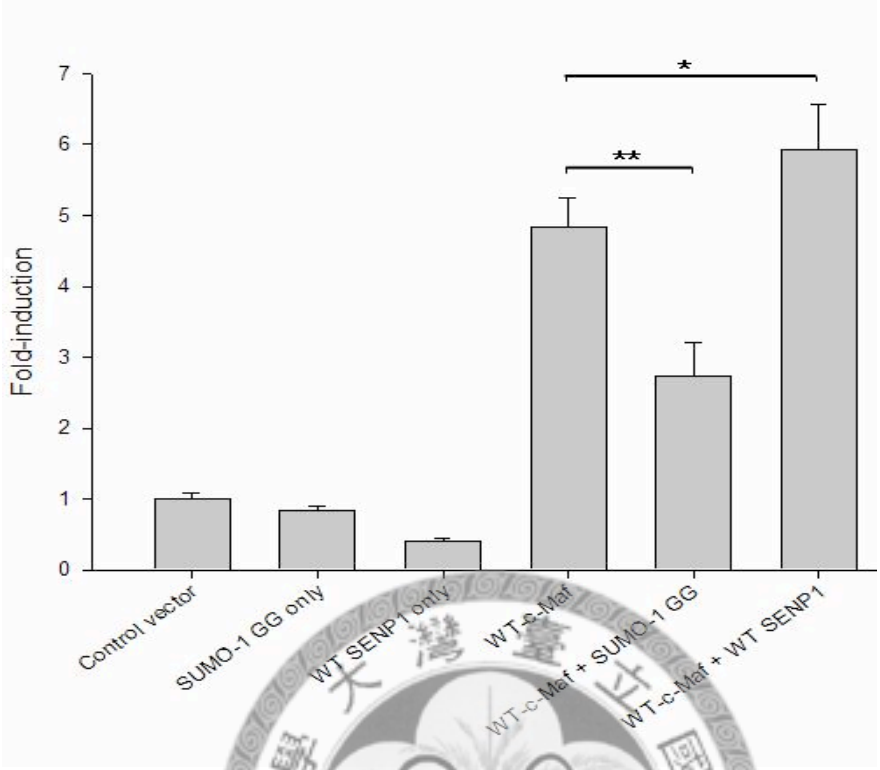
Zhou, L., Ivanov, II, Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D.E., Leonard, W.J., and Littman, D.R. (2007). IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nature immunology *8*, 967-974.

Appendix



APPENDIX FIGURE 1. c-Maf co-localizes with Ubc-9 and PIAS1 in the nucleus. HEK293T cells (4×10^5) were co-transfected with pEYFP-Ubc-9 (green) or pEYFP-PIAS1 (green) and pDsRed-c-Maf (red) expression vectors. Twenty-four hours after transfection, cells were fixed and stained with DAPI (blue) and viewed by confocal microscopy. Co-localization of c-Maf and Ubc-9 or PIAS1 is shown as yellow color in the merged microscopy.

A


Fold-induction
0
1
2
3
4
5
6
7
*
**
Control vector
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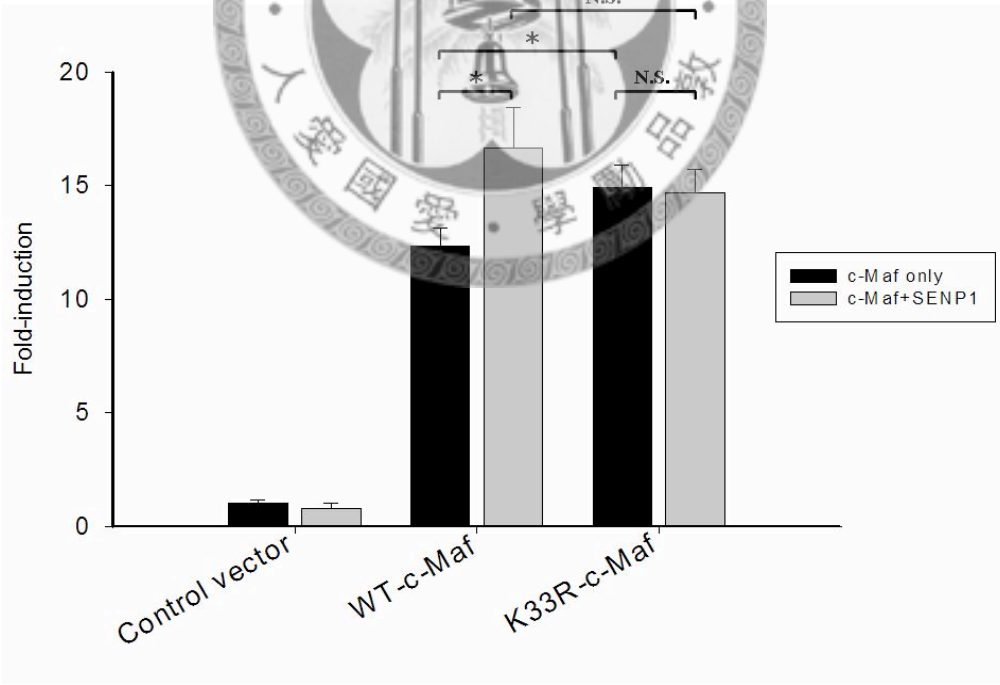
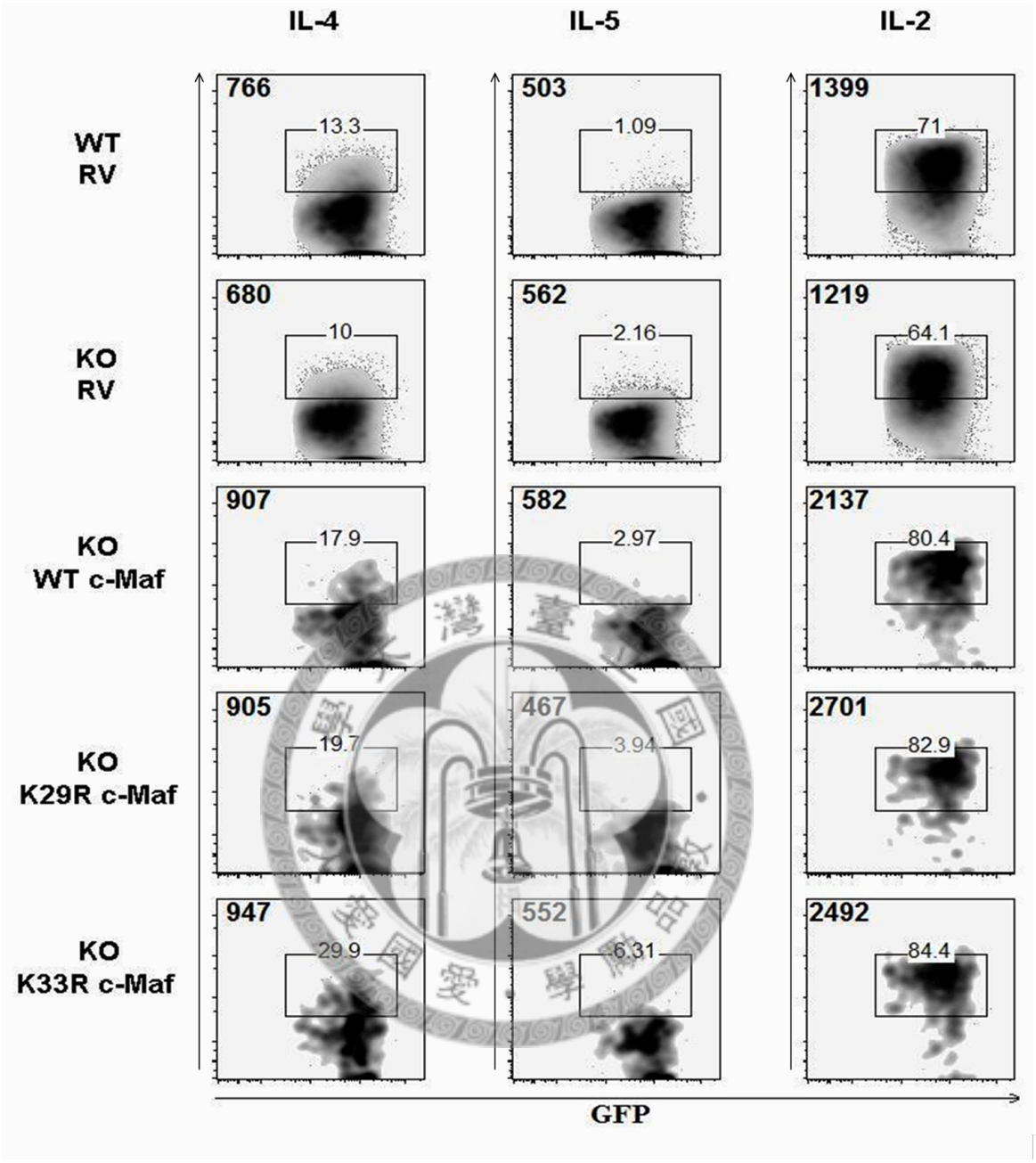
WT SENP1 only
WT-c-Maf
WT-c-Maf + SUMO-1 GG
WT-c-Maf + WT SENP1

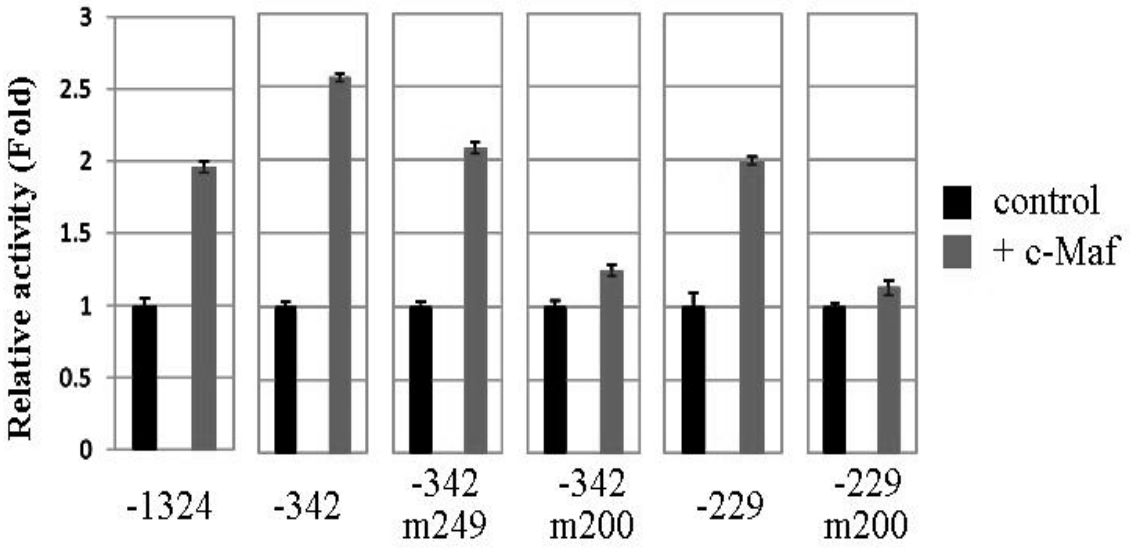
B


Fold-induction
0
5
10
15
20
N.S.
*
*
N.S.
c-Maf only
c-Maf+SENP1
Control vector
WT-c-Maf
K33R-c-Maf

APPENDIX FIGURE 2. The transcription activity of c-Maf is suppressed by SUMOylation. SUMO attenuates but SENP1 enhances WT-c-Maf activity in DO11.10. T cells (2×10^5) were transfected with Renilla, *Il4-promoter* luciferase reporter plasmid, and indicated expression vectors. Cell lysate was analyzed with the Dual-Glo Luciferase Assay System (Promega). *Il4-promoter*-luciferase activity was normalized against renilla luciferase and compared with the normalized activity of the empty control vector, which was arbitrarily set at 1. A fraction of the cell lysate was subjected to immunoblotting with indicated antibody to show equal expression of indicated proteins. Mean and SD values (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.) were obtained from three independent samples (n=3). Each experiment was performed at least three times with similar results.

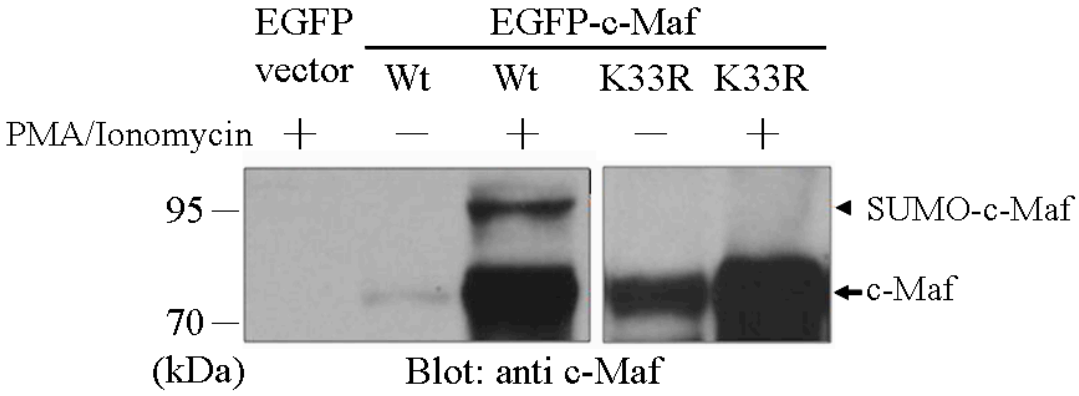

IL-4
IL-5
IL-2
WT RV
KO RV
KO WT c-Maf
KO K29R c-Maf
KO K33R c-Maf
766
13.3
503
1.09
1399
71
680
10
562
2.16
1219
64.1
907
17.9
582
2.97
2137
80.4
905
19.7
467
3.94
2701
82.9
947
29.9
552
6.31
2492
84.4
GFP

APPENDIX FIGURE 3. K33R-c-Maf is more potent than WT-c-Maf in inducing IL-4 production in c-Maf-deficient Th cells. c-Maf-deficient Th cells were obtained as described in Materials and Methods. Cells were activated with anti-CD3 (1 μg/ml)/-CD28 (2 μg/ml) antibodies under a Th0-skewing condition, and then transduced with empty GFPRV or GFPRV-expressing WT, K29R, or K33R-c-Maf. Cells were re-stimulated with PMA/ ionomycin and subjected to intracellular cytokine staining with indicated anti-cytokine antibody. GFP^+ cells were gated and the percentages of cytokine-producing cells are shown. The number shown at the upper-left corner in each panel stands for the MFI of the GFP^+ cells.

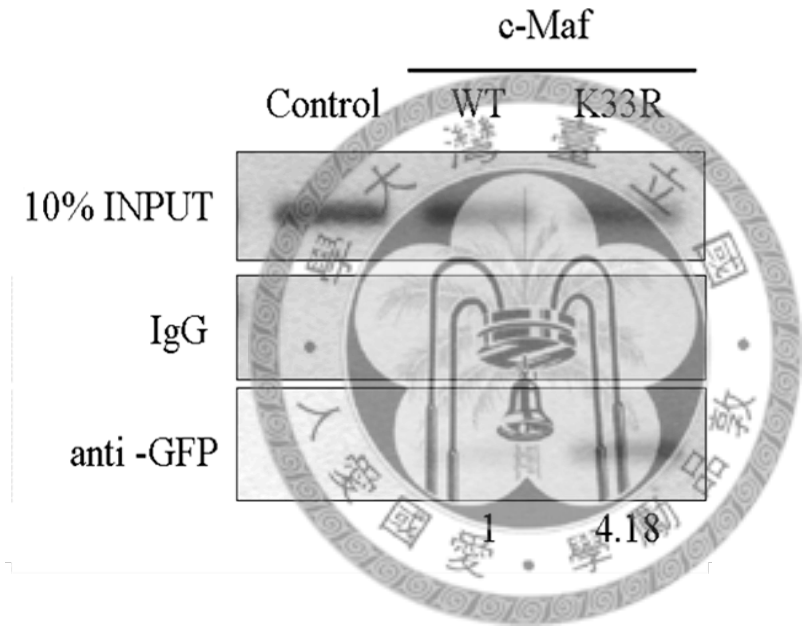


APPENDIX FIGURE 4. Proximal -200 MARE site is essential for c-Maf mediated IL-21 gene expression. HEK293T were transfected with Renilla, IL-21-promoter luciferase reporter plasmid, combined with empty expression vector or c-Maf expression vector for 24 hours. Cell lysate was analyzed with the Dual-Glo Luciferase Assay System (Promega). The luciferase activity was normalized against renilla luciferase and compared with the normalized activity of the empty control vector, which was arbitrarily set at 1. Mean and SD values (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.) were obtained from three independent samples (n=3). Each experiment was performed at least three times with similar results.

A


EGFP
vector
EGFP-c-Maf
Wt
Wt
K33R
K33R
PMA/Ionomycin
+
−
+
−
+
95
70
(kDa)
SUMO-c-Maf
c-Maf
Blot: anti c-Maf

B


c-Maf
Control
WT
K33R
10% INPUT
IgG
anti -GFP
1
4.18

APPENDIX FIGURE 5. SUMOylation affects the recruitment of c-Maf to the IL-4 gene.

(A) WT-c-Maf, but not its K33R-c-Maf mutant, undergoes SUMOylation in DO11.10 cells. DO11.10 cells (2×10^6) were transfected with pEGFP-WT-c-Maf or pEGFP-K33R-c-Maf expression vector. One day after transfection, cells were stimulated with or without PMA (50 ng/ml) and ionomycin (1 μM) for 6 hours. Cell lysates were collected and immunoblotted with anti-c-Maf antibody. The arrow and arrowhead indicate native and SUMO-modified c-Maf, respectively. **(B) K33R-c-Maf is more robustly recruited to the IL-4 gene than WT-c-Maf.** DO11.10 T cells (4×10^5) were transfected with WT- or K33R-EGFP-c-Maf expression vector and stimulated with PMA/Ionomycin. Cells were harvested and subjected to ChIP assay as described in Materials and Methods. The input chromatin was 10% of the total chromatin measured after sonication. The amount of IL-4 gene fragment amplified from WT-c-Maf expressing cells (WT) was arbitrarily set as 1.