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SUMOylation attenuates c-Maf-dependent IL-4 expression

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The function of transcription factors can be critically regulated by SUMOylation. c-Maf, the cellular counterpart of v-maf oncogene, is a potent transactivator of the IL-4 gene in Th2 cells. We found in a yeast two-hybrid screen that c-Maf can interact with Ubc9 and PIAS1, two key enzymes of the SUMOylation pathway. In this study, we report that c-Maf co-localized with these two SUMO (small ubiquitin-like modifier) ligases in the nucleus and that c-Maf can be SUMOylated *in vitro* and also in primary Th2 cells. We also demonstrated that lysine-33 is the dominant, if not the only, SUMO acceptor site of c-Maf. SUMOylation of c-Maf attenuated its transcriptional activity. Reciprocally, a SUMOylation resistant c-Maf was more potent than WT-c-Maf in driving IL-4 production in c-Maf-deficient Th2 cells. Furthermore, we showed that ablation of the SUMO site did not alter the subcellular localization or the stability of c-Maf protein but instead enhanced its recruitment to the *Il4*-promoter. We conclude that SUMOylation at lysine-33 is a functionally critical post-translational modification event of c-Maf in Th cells.

Key words: c-Maf · *Il4* Gene regulation · SUMOylation

Supporting Information available online

Introduction

IL-4, the signature cytokine of type 2 Th (Th2) cells, plays an important role in adaptive immune responses [1, 2]. Previous studies have shown that the expression of IL-4 is critically regulated by c-Maf, a member of AP-1 basic region/leucine zipper transcription factors [3, 4]. Naïve Th cells express little c-Maf, the expression of which is up-regulated by TCR/CD28 signaling during Th2, but not Th1, cell differentiation in a Vav1-dependent manner [5]. c-Maf binds to a half MARE (Maf recognition element) site and transactivates the IL-4 promoter. Forced expression of c-Maf is sufficient to drive endogenous IL-4 production in M12 B cells or differentiating Th1 cells [6, 7]. Reciprocally, c-Maf-deficient Th cells have a profound defect in differentiating into Th2 cells under non-polarizing conditions but are able to become Th2 cells in the presence of exogenous IL-4. Although c-Maf-deficient Th2 cells are capable of producing normal levels of IL-5 and IL-13, they still express a low level of IL-4 [8]. Thus, c-Maf is a potent and specific transactivator of the *Il4* gene.

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The activity of transcription factors is not only controlled by their synthesis but also modulated by complicated post-translational modifications, such as phosphorylation. For example, phosphorylation of v-Maf by GSK3 kinase enhances its transforming activity [9]. In addition to phosphorylation, SUMOylation can critically influence the function of transcription factors. SUMO is a small ubiquitin-like modifier, also known as smt3, sentrin, GMP1 or PIC1 [10–12]. Four SUMO-isoforms, SUMO-1, SUMO-2, SUMO-3 and SUMO-4, exist in mammalian cells. At the protein level, SUMO-1 is only 47% identical to SUMO-2 and SUMO-3, whereas SUMO-2 and SUMO-3 are 95% identical to each other [13]. The 3D structure of SUMO is highly similar to that of ubiquitin [14]. SUMO uses an ubiquitin-like enzyme system to transport and modify its targets. 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 [15]. SUMO-1 conjugates to its targets as a monomer, whereas SUMO-2, SUMO-3 and SUMO-4 can form polymers [16, 17]. SUMO recognize and bind to lysine residues in the context of ΨKXE, where Ψ is a large hydrophobic amino acid and X means any amino acid residue [18]. SUMO affects the function of its target proteins in diverse ways, including changing protein stability, cross-talking with other post-translational modifications, altering protein–protein interaction, changing DNA binding affinity, or altering subcellular localization [19–21].

Two c-Maf-related proteins, MafB and MafA, were reported to be modified and their activity affected by SUMO [22, 23]. But it is unclear whether c-Maf is subjective to SUMOylation and, if so, what the impact of SUMOylation is on the function of c-Maf. Using a yeast two-hybrid system, we identified two SUMO ligases, Ubc9 and PIAS1, which can interact with the N-terminal activation domain (AD) of c-Maf. In this study, we report that c-Maf is modified by SUMO on a single lysine residue *in vitro* and *in vivo*. A lack of SUMO conjugation enhances c-Maf activity on IL-4 gene expression. We further demonstrate that the recruitment of c-Maf to the *Il4*-promoter is modulated by SUMOylation.

Results

c-Maf interacts with PIAS1 and Ubc9

Prior to this study, we carried out a yeast two-hybrid experiment, using the N-terminal 138 amino acids of c-Maf as bait (data not shown) to screen a T-cell cDNA library. We identified both Ubc9 and PIAS1, the SUMO-E2 and SUMO-E3 ligases, as candidates for c-Maf interacting proteins. In this study, we began with examining the intracellular localization of these three molecules. DsRed-c-Maf, EYFP-Ubc9 and EYFP-PIAS1 fusion protein vectors were constructed and introduced into HEK293 T cells. Fluorescence microscopy showed that c-Maf co-localized with Ubc9 and PIAS1 inside the nucleus (indicated by DAPI staining) (Fig. 1A). These data indicate that c-Maf might physically interact with Ubc9 and PIASI in the nucleus and thus be conjugated with SUMO molecule.

Next, we examined whether c-Maf could interact with the SUMO-conjugating enzymes in primary Th cells. Naïve $CD4^+$ T cells were cultured under a 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 (Fig. 1B), demonstrating their physical interaction in primary Th2 cells.

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

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 (Fig. 2A). We first set to study whether c-Maf could be modified by SUMO 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, GST-SUMO-2 or GST-SUMO-3. Figure 2B shows that in the absence of SUMO, only one protein band of approximately 70 kD, representing the unmodified GST-c-Maf, was detected. However, in the presence of SUMO, at least two new protein bands that were recognized by c-Maf antibody appeared. The dominant one was of 120 kD, corresponding to mono-SUMOylated-GST-c-Maf. Protein band of 170 kD was detected only in the presence of SUMO-2 or SUMO-3, probably representing di-SUMOylated-GST-c-Maf.

In vivo SUMOylation of c-Maf was performed in HEK293 T cells. Cells were co-transfected with vectors expressing HA-c-Maf and EGFP-SUMO1-GG (the active form of SUMO), EGFP-SUMO1-AA (the inactive form of SUMO), Ubc9, WT deSUMOylation enzyme SENP1 or SENP1-C603S (an inactive form of SENP1). Immunoblotting with anti-c-Maf antibody expectedly showed that WT-c-Maf was SUMOylated by SUMO-1-GG but not by SUMO-1-AA. Co-expression of SENP1-C603S, but not WT-SENP1, led to SUMOylation of c-Maf even in the absence of exogenous Ubc9 (Fig. 2C, left panel). One possible explanation is that the exogenous c-Maf underwent constant SUMOylation and de-SUMOylation in HEK239 T cells by endogenous SUMO and relevant enzymes. But the level of SUMOylated-c-Maf in the absence of exogenous SUMO and Ubc9 was too low to be detected in this assay. 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 generated a mutant c-Maf, K29,33,328R, in which the lysine residues within the three predicted SUMO-conjugating sites were converted to arginine. In experiments using the mutants, we detected very little, if any at all, SUMOylated-K29,33,328R c-Maf in HEK293 cells (Fig. 2C, right panel).

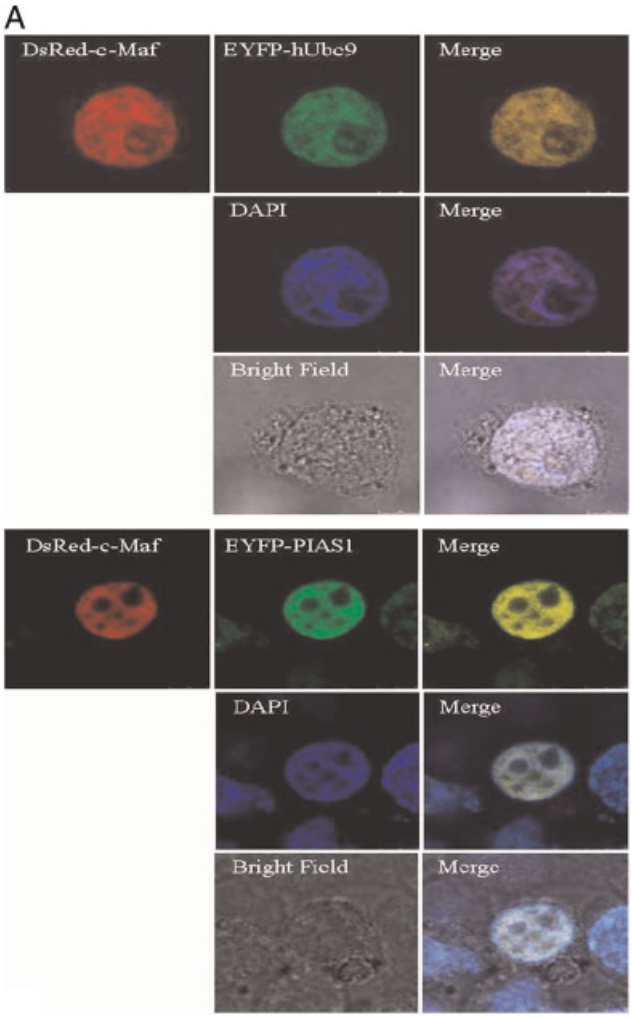

Figure 1. c-Maf interacts with SUMO-E2 and SUMO-E3 ligases. (A) c-Maf co-localizes with Ubc9 and PIAS1 in the nucleus. HEK293 T cells (4×10^5) were co-transfected with pEYFP-Ubc9 (green) or pEYFP-PIAS1 (green) and pDsRed-c-Maf (red) expression vectors. Twenty-four hours after transfection, cells were fixed and stained with DAPI (blue) and viewed by confocal microscopy. Co-localization of c-Maf and Ubc9 or PIAS1 is shown as yellow color in the merged microscopy. (B) 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. Cells were re-stimulated with 1 μg/mL of anti-CD3, then lysed in NP-40 lysis buffer. The whole cell lysate (WCL) was precipitated with anti-c-Maf antibody or control rabbit IgG (Rb Ig) and the immunoprecipitate was eluted and analyzed by Western blotting with anti-PIAS1 or anti-c-Maf antibodies.

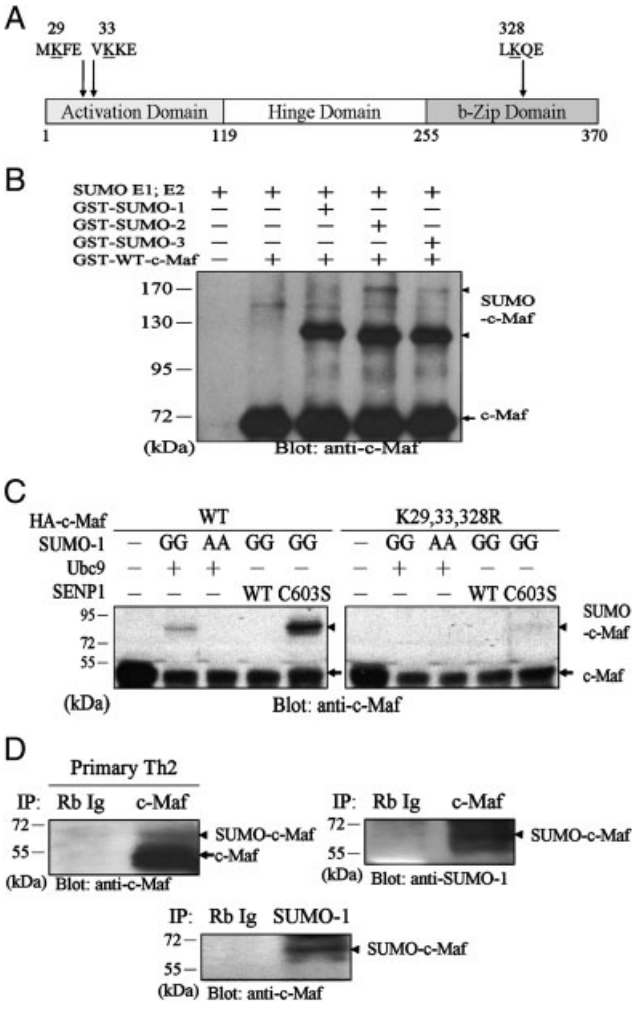

Figure 2. *In vivo* and *in vitro* SUMOylation of c-Maf. (A) A schematic diagram of c-Maf protein. The three predicted SUMO sites are indicated. The numbers represent the numbers of the amino acid residues. (B) *In vitro* SUMOylation of c-Maf. Purified GST-c-Maf was incubated in the reaction mixture containing SUMO-E1, SUMO-E2, ATP and the indicated SUMO at 30°C for 1 h. The reaction mixtures were immunoblotted with anti-c-Maf antibody. (C) *In vivo* SUMOylation of c-Maf in HEK293 T cells. HEK293 T cells (2×10^5) were co-transfected with pcDNA3.1 encoding HA-WT- or HA-K29,33,328R-c-Maf, and the indicated expression vectors. Cells were lysed and the cell lysates were immunoblotted with anti-c-Maf antibody. (D) *In vivo* SUMOylation of c-Maf in primary Th2 cells. Primary cells were restimulated with anti-CD3 antibody for 24 h before lysis by NP-40 lysis buffer containing 20 mM N-ethylmaleimide (NEM). Cell lysate was immunoprecipitated with anti-c-Maf (top) 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 (top-right) or anti-c-Maf (top-left and bottom) antibody. The arrows indicate the original non-SUMOylated-c-Maf and the arrowheads indicate the SUMO-modified-c-Maf.

We 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 immunoprecipitated and detected by anti-c-Maf antibody (Fig. 2D, top-left 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 (Fig. 2D, top-left and top-right panels). Furthermore, anti-SUMO-1 antibody also precipitated a 70-kD protein that was recognized by anti-c-Maf antibody (Fig. 2D, bottom panel). Similar result was obtained when we repeated the experiment in DO11.10 cells (data not shown). These results demonstrate that c-Maf is SUMOylated not only in HEK293 T cells but also in re-activated primary Th2 cells under physiological conditions.

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. We generated three c-Maf mutants: K29R, K33R and K328R, in which lysine at positions 29, 33 and 328 was converted to arginine. To test which mutant lost the ability to conjugate SUMO, each mutant was introduced into HEK293 T cells. *In vivo* SUMOylation assay showed that both K29R- and K328R-cMaf were modified by SUMO as WT-c-Maf was and that ablation of K33 rendered c-Maf completely resistant to SUMOylation even in the presence of exogenous SUMO (Fig. 3A). 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 dominant 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 (Fig. 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. The importance of K33 in the SUMO motif is further illustrated by its conservation among all large Maf family members (Fig. 3C).

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

Previous studies have shown that c-Maf serves as a key regulator of IL-4 production [6, 8]. The next question was whether SUMOylation had any impact on the c-Maf-dependent *Il4* gene expression. HEK293 T cells were transfected with an *Il4*-promoter luciferase reporter along with vectors expressing WT-c-Maf, SUMO-1-GG, SUMO-1-AA, SENP1 and/or SENP1-C603S. As expected, c-Maf transactivated the *Il4*-promoter by nearly 100-fold as compared to vector alone (Fig. 4A). While the inactive SUMO-1-AA did not change the transactivation of *Il4*-promoter by c-Maf ($p = 0.06$, not significant), the presence of SUMO-1-GG reduced the *Il4*-promoter

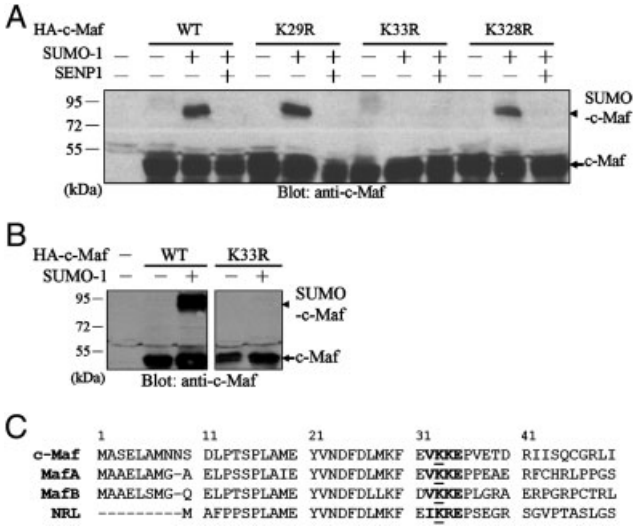

Figure 3. Identification of the SUMO acceptor site(s) in c-Maf. (A) Characterization of c-Maf SUMO-acceptor site. HEK293 T cells (2×10^5) were transfected with pcDNA3.1 encoding HA-WT-, HA-K29R-, HA-K33R- or HA-K328R-c-Maf, combined with the indicated expression vectors. Cells were lysed and immunoblotted with anti-c-Maf antibody. (B) WT-c-Maf, but not K33R-c-Maf mutant, undergoes SUMOylation in DO11.10 cells. 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. (C) 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.

activity by 22% ($p = 0.02$). Reciprocally, SENP1 enhanced c-Maf-induced *Il4*-promoter activity by about 23% ($p = 0.01$), whereas SENP1-C603S further suppressed the c-Maf activity by about 42% ($p = 0.001$). These data indicate that SUMO modification of c-Maf attenuates its ability to transactivate the *Il4*-promoter.

As the K33R mutant of c-Maf was not SUMOylated *in vivo*, we hypothesized that this mutant would be more potent than the WT-c-Maf and that its activity would not be affected by SENP1 *in vivo*. We trasnfected HEK293 T cells with the *Il4*-promoter luciferase reporter vector, a c-Maf (WT, K29R, K33R or K328R) expression vector, and/or the SENP1 expression vector. Luciferase activity assay showed the SUMOylation-resistant K33R mutant was more active than WT-c-Maf ($p = 0.007$), whereas K29R and K328R c-Maf were functionally comparable to WT-c-Maf (Fig. 4B). While co-expression of SENP1 enhanced the activities of WT-, K29R- and K328R-c-Maf ($p = 0.004$, 0.0003 and 0.006, respectively) on *Il4*-promoter activity, it did not enhance that of K33R-c-Maf ($p = 0.953$). Thus, blocking the SUMOylation of c-Maf by either mutating the acceptor site or co-expressing the deSUMOylation enzyme enhances its transactivation of the *Il4*-promoter.

To determine whether the impact of SUMOylation on the transcriptional activity of 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

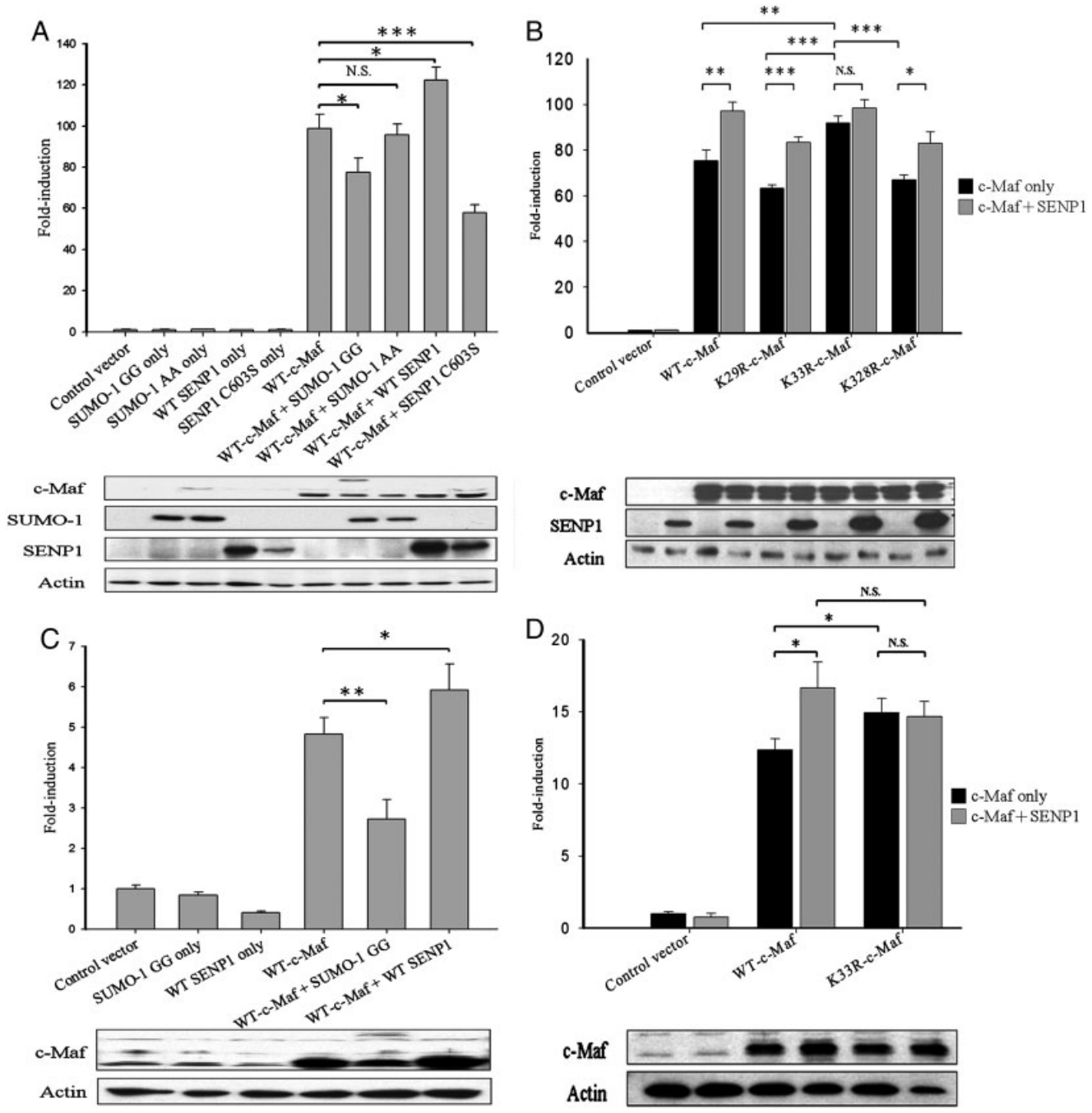

Figure 4. The transcription activity of c-Maf is suppressed by SUMOylation. SUMO attenuates but SENP1 enhances WT-c-Maf activity in HEK293 T cells. (A, B) HEK293 T or (C, D) DO11.10 T cells (2×10^5) were transfected with Renilla, *Il4*-promoter luciferase reporter plasmid, and indicated expression vectors. 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.

also attenuated by SUMO ($p = 0.002$) and enhanced by SENP1 ($p = 0.035$) (Fig. 4C). 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 ($p = 0.019$) (Fig. 4D).

To examine the effect of c-Maf SUMOylation on the endogenous *Il4*-promoter, we 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 mutant c-Maf were separately introduced into $CD4^+$ T cells. 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. 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 threefold (approximately 30%) (Fig. 5). 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 *versus* 2.97%). Although it awaits confirmation, 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 among groups that were transfected with WT-, K29R- and K33R-c-Maf, showing that the expression of c-Maf does not 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.

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

To address the question of how SUMO modification affects the function of c-Maf, we first examined whether c-Maf protein stability is altered by SUMOylation. WT- or K33R-c-Maf and SUMO-1 were co-expressed in HEK293 T cells and the cells were treated with cycloheximide (CHX) to inhibit protein synthesis. Cells were collected at different time points after CHX treatment and c-Maf protein levels were determined. Figure 6A shows that the degradation kinetics of K33R-c-Maf protein coincided with that of the WT-c-Maf, indicating that SUMOylation does not alter the protein stability of c-Maf.

The subcellular localization of WT-c-Maf and its K33R mutant was then analyzed. pEGFP-WT- or pEGFP-K33R-c-Maf fusion

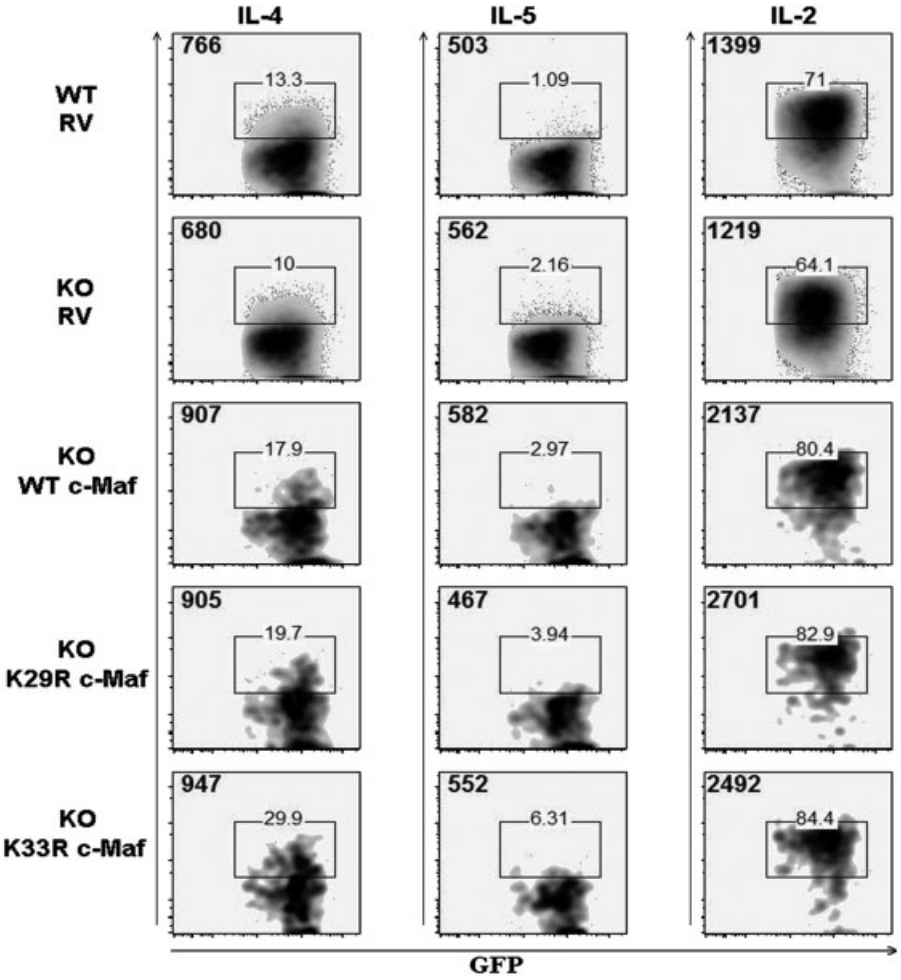

Figure 5. K33R-c-Maf is more potent than WT-c-Maf in inducing IL-4 production in c-Maf-deficient Th cells. c-Maf-deficient Th cells were obtained as described in *Materials and methods*. Cells were activated with anti-CD3 (1 μg/mL)/-CD28 (2 μg/mL) antibodies under a Th0-skewing condition and then transduced 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.

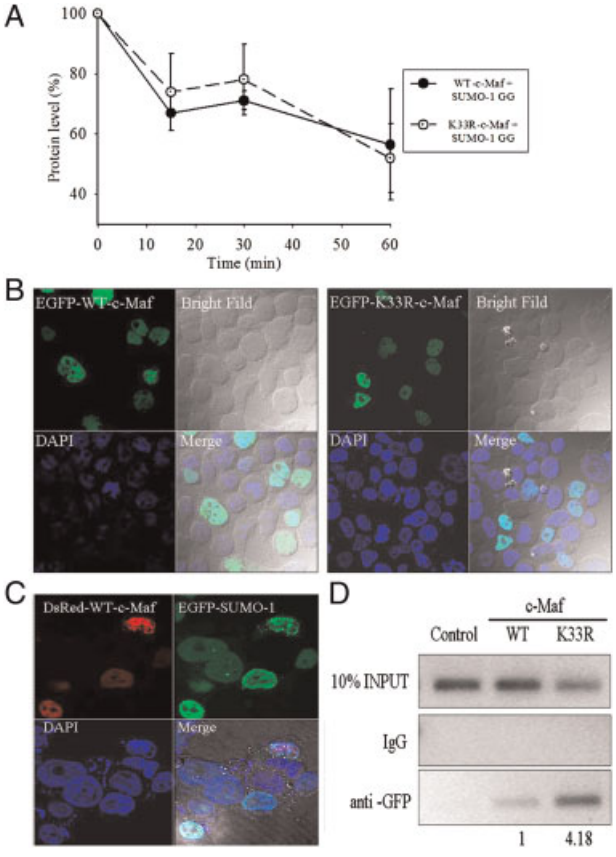

Figure 6. SUMOylation affects the recruitment of c-Maf to the IL-4 gene. (A) SUMOylation does not affect c-Maf protein stability. HEK293 T cells (3×10^6) were co-transfected with EGFP-SUMO-1-GG and a vector expressing HA-tagged WT- or K33R-c-Maf. The stability of c-Maf protein was measured as described in the *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. (B) The cellular localization of WT- and K33R-c-Maf. HEK 293 T cells (4×10^5) were transfected with a vector expressing EGFP-WT- or -K33R-c-Maf, respectively. Cells were fixed and stained with DAPI (blue), and the localization of c-Maf was viewed under a confocal microscope. (C) SUMOylation does not affect the cellular localization of WT-c-Maf. HEK 293 T cells (4×10^5) were transfected with vectors expressing DsRed-c-Maf and EGFP-SUMO-1. The localization of the c-Maf and SUMO-1 were viewed under a confocal microscope. (D) 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.

protein was expressed in HEK293 T cells. Confocal imaging showed that both WT-c-Maf and K33R-c-Maf were located exclusively in the nucleus (Fig. 6B). Furthermore, the exclusive nuclear localization of c-Maf was not altered by overexpression of SUMO. Both c-Maf and SUMO co-localized in the nucleus when overexpressed in HEK293 T cells (Fig. 6C). These data demonstrate that SUMOylation does not alter the subcellular localization of c-Maf.

The next question was whether SUMO modification affects c-Maf recruitment to the endogenous *Il4*-promoter. DO11.10 cells expressing either GFP-WT-c-Maf or GFP-K33R-c-Maf were subjected to chromatin immunoprecipitation (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 (Supporting Information Fig. 1). Interestingly, while both WT- and K33R-c-Maf were recruited to the *Il4*-promoter, the recruitment of K33R-c-Maf was four-times more robust than that of the WT-c-Maf (Fig. 6D). The result strongly indicates that SUMOylation at the K33 site inhibits the activity of c-Maf by attenuating its recruitment to the *Il4*-promoter.

Discussion

In this report, we show that c-Maf is post-translationally modified by SUMO molecules. We also identify K33 as the dominant, if not the only, acceptor site for SUMO and demonstrate that conjugating with SUMO suppresses the transcription activity of c-Maf. Moreover, SUMOylation-resistant c-Maf is more easily recruited to the *Il4*-promoter. Therefore, SUMOylation of c-Maf interferes with its recruitment to the *Il4*-promoter, thereby negatively regulating the production of IL-4.

Our data also lend support to the notion that the function of large Maf proteins is tightly regulated by post-translational modifications. It has been shown that phosphorylation of v-Maf by GSK3 kinase enhances its transforming activity. MafA and MafB, two large Maf proteins, can be phosphorylated by P38 MAP kinase [23, 24]. Phosphorylation of MafA enhances its ubiquitination and increases the recruitment of coactivator P/CAF by MafA [9]. Prior to this report, MafB and MafA have been shown to be modified by SUMO [22]. MafA can be SUMOylated by SUMO-1 and SUMO-2 on lysine 32 residue, and SUMO modification is triggered by hypoglycemia or by oxidative stress. MafB is reported to be SUMOylated by SUMO-1 on lysine 32 and 297 residues *via* v-myb signaling. It is intriguing that these three Maf proteins use a consensus site in the AD for their SUMOylation, *i.e.* the lysine 33 residue of c-Maf, and the lysine 32 residue of MafA and MafB. In addition, the effects of SUMOylation on these three Maf proteins are all negative. SUMOylation of MafA decreases its transcriptional activity on the insulin promoter and intensifies the suppression of CHOP10 expression [23]. SUMO modification suppresses MafB to promote macrophage differentiation and inhibits cell cycle procession [22]. Thus, SUMO modification is a conserved regulatory mechanism for this family of transcription factors.

Some SUMO acceptor sites, such as those of HIC1 and C/EBPδ, overlap with the ψKXEP motif, where the lysine residue can also be acetylated [25, 26]. The SUMO site of MEF2D (myocyte enhancer factor 2D) lies within ΨKXEPXSP motif that can undergo much more complicated switching among acetylation, SUMOylation, and phosphorylation [27, 28]. It is worth mentioning that the amino acid sequences neighboring K33 of c-Maf (and K32 of MafA and MafB) match to the ΨKXEP motif

(Fig. 3C). Whether K33 of c-Maf can also undergo acetylation/SUMOylation switch is an interesting topic.

SUMO modification does not alter the degradation or the subcellular localization of c-Maf. But how does SUMO modification attenuate the recruitment of c-Maf to the IL-4 gene? NRL, also a large Maf protein, can interact with TBP through the minimal transactivation domain) inside the AD region [29]. The K33 locates just right before the minimal transactivation domain (amino acid 47–110 residues) of c-Maf. Thus, the addition of SUMO may physically block the interaction between c-Maf and TBP. SUMO is known to interact with proteins bearing a SUMO interacting motif (SIM) [30]. SUMOylated-c-Maf may be sequestered from the IL-4 promoter by yet-to-be-determined SIM-containing proteins. The dominant SUMO site (K33) is located at the N-terminus, far away from the C-terminal DNA binding region. Thus it is unlikely that SUMO molecules directly block the binding of c-Maf to the half MARE site of the *Il4*-promoter. However, SUMOylation may result in intra-molecular conformational changes of c-Maf, leading to the seclusion of its DNA binding domain. These scenarios are non-mutually exclusive and remain to be clarified.

Other transcription factors, besides c-Maf, can also induce IL-4 production by binding to its promoter. Some of these transcription factors have been reported to be SUMO-modified, but the impact of each of these SUMOylation events on IL-4 production is quite different. For example, JunB, an AP-1 family member, has been shown to be SUMOylated on lysine 237 residue in T cells. Blocking JunB SUMOylation diminishes JunB-mediated IL-2 and IL-4 gene expression [31]. The CCAAT/enhancer-binding protein beta (C/EBPβ) has been reported to transactivate IL-4 gene expression in murine T cells and facilitates Th2 cell responses. SUMO-modification inhibits C/EBPβ-mediated repression of *c-myc* expression but has little effect on IL-4 gene expression [32]. The Th2 master regulator GATA3 can also interact with Ubc9 and PIAS1. Overexpression of PIAS1 enhances GATA3 driven IL-13 expression but does not affect IL-4 production [33]. NFAT and YY1 have also been documented to be SUMO-modified, but the effect of SUMO modification on their IL-4 induction abilities has not yet addressed [34, 35]. As these transcription factors are often simultaneously induced during the differentiation of Th2 cells, these SUMOylation events have to be coordinated in a way to achieve the optimal production of IL-4. A recent publication reported that the binding of c-Maf to the IL-4 promoter was impaired in Th cells obtained from NOD mice [36]. They concluded that the impaired binding was due to enhanced SUMOylation of c-Maf at K33. As NOD Th cells display a defect in IL-4 production, it will be of great interest to examine the status of post-translational modifications, including SUMOylation, of the relevant transcription factors in NOD Th cells.

In addition to transactivating the *Il4* gene, c-Maf also plays important roles in both adaptive and innate immunity *via* IL-4-independent mechanisms. c-Maf is required for the optimal induction of CD25 in developing Th2 cells [37]. Recently, ICOS signal has been found to induce c-Maf expression on interleukin-17 (IL-17)-producing Th cells (Th17 cells) and follicular Th cells. Deficient c-Maf results in a decrease in the production of IL-21 and expansion of Th-17 and follicular Th cells [38]. c-Maf also regulates the expression of IL-10 and IL-12 receptor gene expressions in monocytes and macrophages [39, 40]. Furthermore, aberrant expression of c-Maf can be detected in myeloma and lymphoma cells, in which it controls the expression of cyclin D2 [41, 42]. Outside the immune system, c-Maf is indispensable for the transcription of crystallin genes and the development of lens [43]. Elucidating the impact of SUMOylation of c-Maf on the expression of c-Maf target genes within or outside the immune system will greatly advance our understanding of the role and mechanism of action of this important transcription factor. This issue is now being addressed.

Materials and methods

Cell cultures and mice

All cells were maintained in either 10 mM HEPES buffered DMEM or in RPMI1640 medium supplemented with 10% FBS, 1% L-glutamine, 1 mM sodium pyruvate, 1 × non-essential amino acid, 100 units/mL streptomycin and penicillin. These reagents were obtained from Invitrogen (Grand Island, NY, USA). c-Maf-deficient mice were generated as described [8]. The number of c-Maf-deficient mice was amplified by reconstitution of BALB/c RAG2-deficient mice with fetal liver cells from N5 BALB/c c-Maf-deficient mice. The animal experiments were approved by the Animal Use Committee in National Taiwan University College of Medicine.

ChIP assay

DO11.10 T cells (2×10^7) were transfected with pEGFP-N1, pEGFP-WT-c-Maf or pEGFP-K33R-c-Maf. Forty-eight hours after transfection, cells were lysed and subjected to ChIP assay. Briefly, the associated EGFP-c-Maf-DNA complexes were immunoprecipitated with anti-EGFP antibody (Abcam, Cambridge, MA, USA)-conjugated agarose beads (Millipore, Temecula, CA, USA). Precipitated samples were collected and the specific region containing the MARE sequence in the *Il4*-promoter was amplified using PCR. Sequences of primers used for PCR were 5-ATTATGGTGTAATTTCCTATGCTG-3 and 5-TTATCAAGAGATGCTAACAATGC-3. PCR products were analyzed in 2% agarose gels.

Confocal microscopy

HEK293 T Cells (4×10^5 cells/well) were cultured on glass cover slips in six-well culture plates containing 10% FBS DMEM for 24 h and were transfected with various expression vectors. Twenty-four hours after transfection, cells were fixed and stained with DAPI, and the samples were then analyzed with a Leica confocal microscope.

Immunoprecipitation assay

For the co-immunoprecipitation assay, Th2 cells were stimulated with anti-CD3 antibody for 24 h and then lysed with NP-40 lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40) supplemented with 20 mM NEM (Sigma, St. Louis, MO, USA), PMSF (Sigma) and a 1% protease inhibitor cocktail (Sigma). Five-hundred microgram cell lysate was incubated with anti-c-Maf antibody (clone M-153, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C on a rotor over night before protein G sepharose beads (Millipore) were added and incubated for another 1 h. 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, naive $CD4^+$ T cells obtained from BALB/c mice were cultured under a Th2-skewing condition. Four days later, the cells were re-stimulated with anti-CD3 antibody (1 μg/mL) for 24 h, then collected and lysed by NP-40 lysis buffer containing 20 mM *N*-ethylmaleimide (NEM; Sigma), the deSUMOylation inhibitor. Five-hundred microgram of cell lysate was immunoprecipitated and blotted with anti-c-Maf and anti-SUMO antibodies, respectively, or *vice versa*.

In vitro Th2 cell differentiation/Intracellular cytokine staining

In vitro Th2 cell differentiation and intracellular cytokine staining were performed according to the protocol described in [44].

In vivo and *in vitro* SUMOylation assay

HEK293 T (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, pEF1α-SENP1-3xFlag (WT) or pEF1α-SENP1 C603S-3xFlag (catalitic site mutant). Cells were lysed 24 h after transfection with NP-40 lysis buffer (50 mM Tris (pH 7.4), 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 h. Reaction products were analyzed using immunoblotting with anti-c-Maf antibody.

Luciferase assay

Combinations of HA-WT- or mutated-c-Maf, EGFP-SUNO-1-GG or -AA mutant, Flag-WT or -mutant SENP-1, *Il4*-promoter luciferase reporter and Renilla vectors were co-transfected into HEK293 T or DO11.10 cells. Twenty-four hours after transfection, cells were collected and analyzed with the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activities were normalized against Renilla luciferase levels.

Protein stability assay

HEK293 T cells (3×10^6) were cotransfected with WT- or K33R-HA-c-Maf and EGFP-SUMO-1-GG on 9-cm dishes, respectively. Twenty-four hours after transfection, cells were subcultured in 12-well culture plates and treated with 20 μg/mL CHX (Sigma). Protein samples were collected after CHX treatment for indicted periods of time and immunoblotted with anti-c-Maf and anti-actin (Millipore) antibodies. 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.

Retroviral constructs and transduction

The WT- and the SUMO acceptor site mutated-c-Maf were cloned into GFPRV vectors. In retroviral transduction experiments, primary $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies under a Th0 condition. The cells were restimulated with anti-CD3 antibody (1 μg/mL) for 30 h retrovirally tranduced with the c-Maf-carrying GFPRV.

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 asterisks (*). $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

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References

1 **Glimcher, L. H. and Murphy, K. M.,** Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes Dev.* 2000. **14**: 1693–1711.

2 **Ho, I. C. and Glimcher, L. H.,** Transcription: tantalizing times for T cells. *Cell* 2002. **109**: S109–120.

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

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

5 **Tanaka, Y., So, T., Lebedeva, S., Croft, M. and Altman, A.,** Impaired IL-4 and c-Maf expression and enhanced Th1-cell development in Vav1-deficient mice. *Blood* 2005. **106**: 1286–1295.

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

7 **Ho, I. C., Lo, D. and Glimcher, L. H.,** c-maf promotes T helper cell type 2 (Th2) and attenuates Th1 differentiation by both interleukin 4-dependent and -independent mechanisms. *J. Exp. Med.* 1998. **188**: 1859–1866.

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

9 **Rocques, N., Abou Zeid, N., Sii-Felice, K., Lecoin, L., Felder-Schmittbuhl, M. P., Eychene, A. and Pouponnot, C.,** GSK-3-mediated phosphorylation enhances Maf-transforming activity. *Mol. Cell* 2007. **28**: 584–597.

10 **Melchior, F.,** SUMO – nonclassical ubiquitin. *Annu. Rev. Cell Dev. Biol.* 2000. **16**: 591–626.

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

12 **Seeler, J. S. and Dejean, A.,** Nuclear and unclear functions of SUMO. *Nat. Rev. Mol. Cell Biol.* 2003. **4**: 690–699.

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

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

15 **Zhao, J.,** Sumoylation regulates diverse biological processes. *Cell Mol. Life Sci.* 2007. **64**: 3017–3033.

16 **Tatham, M. H., Jaffray, E., Vaughan, O. A., Desterro, J. M., Botting, C. H., Naismith, J. H. and Hay, R. T.,** Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J. Biol. Chem.* 2001. **276**: 35368–35374.

17 **Bohren, K. M., Nadkarni, V., Song, J. H., Gabbay, K. H. and Owerbach, D.,** 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.* 2004. **279**: 27233–27238.

18 **Johnson, E. S.,** Protein modification by SUMO. *Annu. Rev. Biochem.* 2004. **73**: 355–382.

19 **Dohmen, R. J.,** SUMO protein modification. *Biochim. Biophys. Acta* 2004. **1695**: 113–131.

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

21 **Liu, B. and Shuai, K.,** Regulation of the sumoylation system in gene expression. *Curr. Opin. Cell Biol.* 2008. **20**: 288–293.

22 **Tillmanns, S., Otto, C., Jaffray, E., Du Roure, C., Bakri, Y., Vanhille, L. *et al.*,** SUMO modification regulates MafB-driven macrophage differentiation by enabling Myb-dependent transcriptional repression. *Mol. Cell Biol.* 2007. **27**: 5554–5564.

23 **Shao, C. and Cobb, M. H.,** Sumoylation regulates the transcriptional activity of MafA in pancreatic beta cells. *J. Biol. Chem.* 2009. **284**: 3117–3124.

24 **Sii-Felice, K., Pouponnot, C., Gillet, S., Lecoin, L., Girault, J. A., Eychene, A. and Felder-Schmittbuhl, M. P.,** MafA transcription factor is phosphorylated by p38 MAP kinase. *FEBS Lett.* 2005. **579**: 3547–3554.

25 **Stankovic-Valentin, N., Deltour, S., Seeler, J., Pinte, S., Vergoten, G., Guerardel, C., Dejean, A. and Leprince, D.,** An acetylation/deacetylation-SUMOylation switch through a phylogenetically conserved psiKXEP motif in the tumor suppressor HIC1 regulates transcriptional repression activity. *Mol. Cell Biol.* 2007. **27**: 2661–2675.

26 **Wang, J. M., Ko, C. Y., Chen, L. C., Wang, W. L. and Chang, W. C.,** Functional role of NF-IL6beta and its sumoylation and acetylation modifications in promoter activation of cyclooxygenase 2 gene. *Nucleic Acids Res.* 2006. **34**: 217–231.

27 **Gregoire, S., Tremblay, A. M., Xiao, L., Yang, Q., Ma, K., Nie, J., Mao, Z. *et al.*,** Control of MEF2 transcriptional activity by coordinated phosphorylation and sumoylation. *J. Biol. Chem.* 2006. **281**: 4423–4433.

28 **Gregoire, S. and Yang, X. J.,** Association with class IIa histone deacetylases upregulates the sumoylation of MEF2 transcription factors. *Mol. Cell Biol.* 2005. **25**: 2273–2287.

29 **Friedman, J. S., Khanna, H., Swain, P. K., Denicola, R., Cheng, H., Mitton, K. P., Weber, C. H. *et al.*,** The minimal transactivation domain of the basic motif-leucine zipper transcription factor NRL interacts with TATA-binding protein. *J. Biol. Chem.* 2004. **279**: 47233–47241.

30 **Hecker, C. M., Rabiller, M., Haglund, K., Bayer, P. and Dikic, I.,** Specification of SUMO1- and SUMO2-interacting motifs. *J. Biol. Chem.* 2006. **281**: 16117–16127.

31 **Garaude, J., Farras, R., Bossis, G., Charni, S., Piechaczyk, M., Hipskind, R. A. and Villalba, M.,** SUMOylation regulates the transcriptional activity of JunB in T lymphocytes. *J. Immunol.* 2008. **180**: 5983–5990.

32 **Berberich-Siebelt, F., Berberich, I., Andrulis, M., Santner-Nanan, B., Jha, M. K., Klein-Hessling, S., Schimpl, A. and Serfling, E.,** SUMOylation interferes with CCAAT/enhancer-binding protein beta-mediated c-myc repression, but not IL-4 activation in T cells. *J. Immunol.* 2006. **176**: 4843–4851.

33 **Zhao, X., Zheng, B., Huang, Y., Yang, D., Katzman, S., Chang, C., Fowell, D. and Zeng, W. P.,** Interaction between GATA-3 and the transcriptional coregulator Pias1 is important for the regulation of Th2 immune responses. *J. Immunol.* 2007. **179**: 8297–8304.

34 **Terui, Y., Saad, N., Jia, S., McKeon, F. and Yuan, J.,** Dual role of sumoylation in the nuclear localization and transcriptional activation of NFAT1. *J. Biol. Chem.* 2004. **279**: 28257–28265.

35 **Deng, Z., Wan, M. and Sui, G.,** PIASy-mediated sumoylation of Yin Yang 1 depends on their interaction but not the RING finger. *Mol. Cell Biol.* 2007. **27**: 3780–3792.

36 **Leavenworth, J. W., Ma, X., Mo, Y. Y. and Pauza, M. E.,** SUMO conjugation contributes to immune deviation in nonobese diabetic mice by suppressing c-Maf transactivation of IL-4. *J. Immunol.* 2009. **183**: 1110–1119.

37 **Hwang, E. S., White, I. A. and Ho, I. C.,** An IL-4-independent and CD25-mediated function of c-maf in promoting the production of Th2 cytokines. *Proc. Natl. Acad. Sci. USA* 2002. **99**: 13026–13030.

38 **Bauquet, A. T., Jin, H., Paterson, A. M., Mitsdoerffer, M., Ho, I. C., Sharpe, A. H. and Kuchroo, V. K.,** 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. *Nat. Immunol.* 2009. **10**: 167–175.

39 **Cao, S., Liu, J., Chesi, M., Bergsagel, P. L., Ho, I. C., Donnelly, R. P. and Ma, X.,** Differential regulation of IL-12 and IL-10 gene expression in

macrophages by the basic leucine zipper transcription factor c-Maf fibrosarcoma. *J. Immunol.* 2002. **169**: 5715–5725.

40 **Homma, Y., Cao, S., Shi, X. and Ma, X.,** The Th2 transcription factor c-Maf inhibits IL-12p35 gene expression in activated macrophages by targeting NF-kappaB nuclear translocation. *J. Interferon Cytokine Res.* 2007. **27**: 799–808.

41 **Hurt, E. M., Wiestner, A., Rosenwald, A., Shaffer, A. L., Campo, E., Grogan, T. *et al.*,** Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. *Cancer Cell* 2004. **5**: 191–199.

42 **Morito, N., Yoh, K., Fujioka, Y., Nakano, T., Shimohata, H., Hashimoto, Y., Yamada, A. *et al.*,** Overexpression of c-Maf contributes to T-cell lymphoma in both mice and human. *Cancer Res.* 2006. **66**: 812–819.

43 **Kim, J. I., Li, T., Ho, I. C., Grusby, M. J. and Glimcher, L. H.,** Requirement for the c-Maf transcription factor in crystallin gene regulation and lens development. *Proc. Natl. Acad. Sci. USA* 1999. **96**: 3781–3785.

44 **Miaw, S. C., Choi, A., Yu, E., Kishikawa, H. and Ho, I. C.,** ROG, repressor of GATA, regulates the expression of cytokine genes. *Immunity* 2000. **12**: 323–333.

Abbreviations: **AD:** activation domain · **ChIP:** chromatin immunoprecipitation · **CHX:** cycloheximide · **MARE:** Maf recognition element · **SIM:** SUMO interacting molif · **SUMO:** small ubiquitin-like modifier · **Th2:** type 2 Th

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