# Sumoylation Modulates NFATc1-mediated Lymphokine Gene Expression

**Dissertation for the completion of doctorate degree in Natural Sciences at the Julius-Maximilians-Universität Würzburg**


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# Acknowledgements

First of all, I would like to thank my supervisor, Dr. Friederike Berberich-Siebelt for the excellent step-by-step guidance, for being available all the time for discussions and most importantly, her personal care for me. She always helped me to stay focused on my study. No words can explain how nicely she encouraged me after each and every failure throughout my stay as a PhD student with her. Her critical comments to my results and suggestion for future experiments were very helpful to execute my study in an ordered manner. Apart from scientific front, I learnt some crispy German vocabulary and a great glimpse of German culture from her.

I would like to thank my Doctor father Prof. Dr. Edgar Serfling, for providing me an opportunity to work in his laboratory. His friendly and constructive criticism had immense impact to refine my experiments. It was really great to work with a fun loving and enthusiastic person like him.

I am thankful to my PhD committee member PD. Dr. Robert Hock for being helpful and available for answering all kinds of questions that were put to him.

I thank to Prof. Dr. Thomas Hünig, the speaker of our graduate college GK-520, for providing my fellowship. During our graduate college course work, I really enjoyed his understanding and explanations of various subjects in Immunology. I admit that, he sparked a great interest in my mind to know further about regulation of immune cell cross talk and function.

Dr. Mindaugas Andrulis and Herr Ralf Kielenbeck, without whom I might not have able to know the strong advantage of bio-imaging techniques like confocal microscopy. I am grateful to them for their hands-on teaching about confocal microscopy system.

For an easy start with my PhD work in this lab, I acknowledge my sincere thank to all my lab members, Stefan in particular, who always had time for discussion, argument and counter-argument to make my data more catchy and comprehensive.

I cannot forget the contribution of my all-time friends Amiya, Rene, Timm and Nikola who always provided me scientific as well as moral support during my stay in this lab. From Amiya, I learnt a lot of fundamental immunology, which helped me to ask correct questions for the correct experiments.

Melanie Schott, my good friend, supported a lot with her skillful technical assistance. Because of her friendly and jovial nature, our working place was always very lively and full of fun. I am deeply thankful for her technical support to my study and most importantly her personal care. I am also thankful to Doris and Ilona for their excellent technical support.

Most of all, I would like to thank my father, Mr. Uday Chand Nayak, my mother Mrs. Sikha Nayak and rest of my family for their endless love, encouragement and support throughout my studies without which it would not be possible to reach this level.

I express my special gratitude to my uncle, Mr. D. D. Nayak and Mr. M. C. Nayak who created a quest for science, in my mind, at the very beginning of my academic career.

I sincerely thank to all of my teachers at school, college and university level, who taught me very basic regulation and evolution of dynamic biological system.

Finally, and most importantly, my special thank to my wife, Mamta. Throughout the period of my PhD study she was my source of inspiration, encouragement and enthusiasm. I admire her knowledge and scientific discussions, which were in many ways, helpful to my work. In a single line, without her love and care, it was just impossible to carry out my study.

I dedicate my thesis to my family, particularly my wife and my teachers.

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## Summary

Sumoylation of transcription factors modulate their activity (either upregulating or downregulating) by altering protein-protein interactions as well as subcelluar/subnuclear localization. The transcription factor family of NFAT (Nuclear Factor of Activated T cells) plays an important role in cytokine gene regulation in T cells. Due to alternative usage of two promoters (P1 & P2), two polyadenylation sites (pA1 and pA2) and alternative splicing events, NFATc1 is expressed in six isoforms which are NFATc1/αA, βA, αB, βB, αC and βC, where α and β refer to two different 1st exons and A, B, C to the differentially spliced and extended C-termini. The short isoforms of NFATc1 (NF-ATc1/A) contain a relatively short C terminus whereas, the longer isoforms, B and C, span the extra C-terminal peptides of 128 and 246 aa, respectively. To analyze the specific biological effects of NFATc1 isoform, a yeast two hybrid screening of a human spleen cDNA library with extra C-terminal peptide of NFATc1 as a bait, was performed. At the end of the assay, the proteins involved in the sumoylation pathway such as Ubc9, PIAS1 were detected with highest frequencies and subsequently were were able to demonstrate that NFATc1 is sumoylated. The extent of sumoylation is isoform specific. While NFATc1/A, harboring only one sumoylation site shows very weak sumoylation, the two additional sites within NFATc1/C lead to efficient sumoylation. This modification directs NFATc1/C into SUMO-1 bodies, which in turn colocalize with PML-nbs. Furthermore, sumoylated NFATc1/C recruits the transcriptional co-repressors HDAC (both class I as well as class II HDACs) which results in a significant decrease of the level of histone acetylation on the IL-2 promoter, an important NFATc1 target gene. As a consequence of this, a decrease of IL-2 production was observed, while NFATc1/C, which can no longer be sumoylated due to mutating the target lysines, exhibited dramatic elevated transcriptional potential on the IL2 promoter. This supports our finding from IL-2 promoter-driven reporter gene assay, which shows downregulation of NFATc1/C transactivation upon sumoylation. Hence, sumoylation exerts a negative effect on NFATc1 transcriptioanl activity. Immunofluorescence studies showed SUMO modification to relocate NFATc1/C also into transcriptionally inactive heterochromatin regions, demonstrated by H3K9 m3 (tri-methylated histone lysine 9) co-localization studies. Interestingly,

in the absence of sumoylation, NFATc1 was partially colocalized with transcriptional hotspots in the nucleus, which might contribute to the higher transcription potentiality of the non-sumoylated NFATc1. It is important to note that, the transcriptional activity of other NFATc1 target genes (IL-13, IFN-γ etc.) was positively upregulated upon sumoylation of NFATc1, suggesting a non-universal effect of sumoylation on NFATc1/C function.

In conclusion, sumoylation directs NFATc1 into nuclear bodies where it interacts with transcriptional co-repressors and relocalize itself with heterochromatin, leading to repression of NFATc1/C-mediated transcription. Most importantly, the effect of NFATc1/C sumoylation is promoter specific. Taken together, SUMO modification alters the function of NFATc1 from an activator to a site-specific transcriptional repressor. This study unraveled a novel regulatory mechanism, which controls isoform specific NFATc1 function.

# Zusammenfassung

Die Aktivität von Transkriptionsfaktoren kann durch die Modifikation mit SUMO positiv oder negativ beeinflusst werden, indem Protein-Protein-Interaktionen als auch die subzelluläre bzw. subnukleäre Lokalisation verändert werden. In T-Zellen spielt die Familie der NFAT (Nuclear Factor of Activated T cells)-Transkriptionsfaktoren eine wichtige Rolle bei der Zytokingenregulation. NFATc1 wird durch die Verwendung zwei verschiedener Promotoren (P1 & P2) bzw. Polyadenylierungsstellen (pA1 & pA2) und alternativen Spleißens in sechs Isoformen exprimiert. Sie werden als NFATc1/aA, bA, aB, bB, aC und bC bezeichnet, wobei a und b sich auf die beiden unterschiedlichen 1. Exons und A, B, C sich auf die differentiell gespleißten und unterschiedlich langen C-Termini beziehen. Die NFATc1/A-Isoformen umfassen einen relativ kurzen C-Terminus, während die langen Isoformen B und C extra-C-terminale Peptide von 128 bzw. 246 Aminosäuren aufweisen. Um die spezifischen, biologischen Effekte der NFATc1-Isoformen zu untersuchen, wurde ein sog. ‚Yeast two Hybrid screen' mit einer humanen Milz-cDNA-Bibliothek und dem NFATc1/C-spezifischen C-Terminus durchgeführt. Am Ende wurden Ubc9 und PIAS1, Proteine, die an der Sumoylierung beteiligt sind, am häufigsten dedektiert. Anschließend konnte gezeigt werden, dass NFATc1 tatsächlich sumoyliert wird. Das Ausmaß an Sumoylierung ist Isoformen abhängig. Während NFATc1/A, das eine einzige Sumoylierungsstelle besitzt, nur eine geringe Sumoylierung aufweist, führen die beiden zusätzlichen Stellen in NFATc1/C zu einer effizienten Modifikation mit SUMO. Diese C-terminale Modifikation dirigiert NFATc1/C in SUMO-1-Körperchen, die mit PML-nbs kolokalisieren. Darüber hinaus rekrutiert sumoyliertes NFATc1/C die transkriptionellen Korepressoren HDAC (sowohl Klasse I wie Klasse II HDACs), was zu einer signifikanten Verringerung der Histonazetylierung am IL-2-Promotor, eines wichtigen NFATc1-Zielgens, führt. Konsequenterweise wurde eine Verminderung der IL-2-Produktion beobachtet, während NFATc1/C, das wegen Mutation der entscheidenden Lysine nicht mehr sumoyliert werden kann, ein dramatisch erhöhtes Transaktivierungspotential am IL-2-Promotor aufwies. Das unterstützt unsere Daten, die mit einem IL-2-Promotor getriebenen Reporterassay gewonnen wurden und zeigen, dass das Transaktivierungspotential von NFATc1/C durch Sumoylierung herabgesetzt

wird. Demzufolge übt Sumoylierung einen negativen Effekt auf die transkriptionelle NFATc1-Aktivität aus. Immunfluoreszenzversuche zeigten, dass die Modifikation mit SUMO außerdem zur Relokalisation von NFATc1/C in transkriptionell inaktive, heterochromatische Regionen führt, was durch die Färbung von trimethyliertem Histon mit anti-H3K9 m3 nachgewiesen wurde. Interessanterweise war in Abwesenheit von Sumoylierung NFATc1 teilweise mit transkriptionellen Hotspots im Kern lokalisiert. Das mag zu dem höheren Transkriptionspotential des nicht-sumoylierten NFATc1 beitragen. Es ist wichtig zu erwähnen, dass die transkriptionelle Aktivität auf andere NFATc1-Zielgene durch die Sumoylierung von NFATc1 positiv verstärkt war. Dies deutet auf einen nicht-universalen Effekt der Sumoylierung auf die NFATc1/C-Funktion hin.

Demzufolge dirigiert Sumoylierung NFATc1 in Kernkörperchen, wo es mit transkriptionellen Korepressoren interagiert und selbst ans Heterochromatin relokalisiert, was zu einer Repression der NFATc1/C vermittelten Transkription führt. Als sehr wichtig erscheint, dass der Effekt der NFATc1/C-Sumoylierung Promotor spezifisch ist. Zusammengenommen verändet die Modifikation mit SUMO die NFATc1-Funktion von einem Transaktivator zu einem DNA-Bindungsstellen spezifischen Repressor. Daher wird hier ein neuer regulatorischer Mechanismus aufgezeigt, der die Isoform spezifische NFAT-Funktion kontrolliert.

# 1. INTRODUCTION

## *NFAT family of transcription factors*

### Overview

NFAT (Nuclear Factor of Activated T cells) was discovered as a putative transcription factor in activated Jurkat T leukemia cells, which binds to the human IL-2 promoter (Shaw et al., 1988 ). Although the NFAT family of transcription factors plays an important and central role during T cell activation, the members are also expressed in other classes of immune cells (Rao et al., 1997). Recent studies established the expression of NFATs in non-immune cells such as skeletal muscle, osteoclasts, cardiac muscle etc. (Hogan et al., 2003).

**NFAT family members**

Five different NFAT family members have been identified i.e. NFATc1, c2, c3, c4 and NFAT5 (Rao et al., 1997). While NFATc1-c4 share a highly homologous (70%) Rel similarity domain (RSD), NFAT5 shares less than 50% RSD homology with other members of NFAT proteins (Lopez-Rodriguez et al., 1999).

Due to alternative usage of two promoter (P1 & P2), two polyadenylation sites (pA1 and pA2) and alternative splicing events, NFAT family members are expressed in multiple isoforms (Chuvpilo et al., 1999). NFATc1 is expressed in six isoforms which are NFATc1/αA, βA, αB, βB, αC and βC, where α and β refer to two different 1$^{st}$ exons and A, B, C to the differentially spliced and extended C-termini (Fig-1.1). As compared to the short isoforms of NFATc1 (NFATc1/A), the longest isoform (NFATc1/C) contains an extra C- terminal peptide that is 246-aa residues long (Chuvpilo et al., 1999). In naive T cells, the longer isoforms (NFATc1/βB and NFATc1β/C) are predominantly expressed. Upon activation, strong expression of the short isoform NFATc1α/A can be observed in effector T cells.

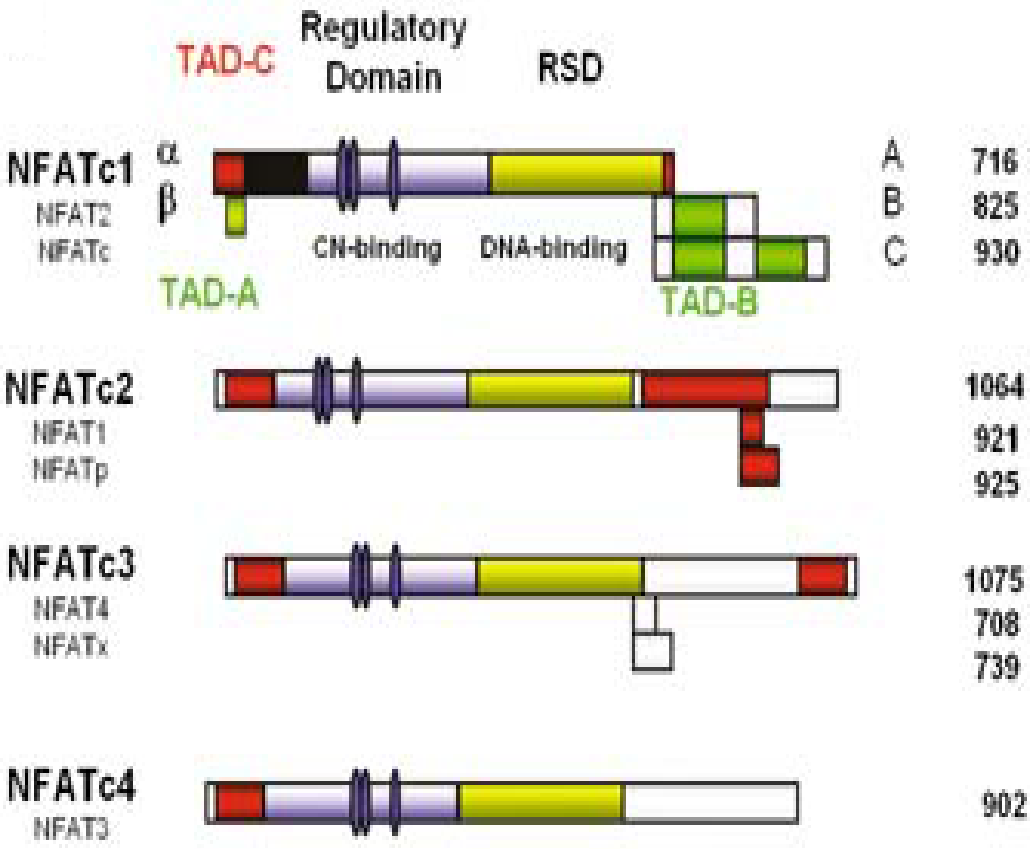



***Fig-1.1* Schematic representation of the family of NFAT transcription factors**. The DNA binding domain (RSD) of NFATs are shown as yellow boxes and the transactivation domains are in red and green. Different NFATc1 isoforms are described. The regulatory domain contains a serine-rich region (SRR), SP-motifs as well as calcineurin binding sites (Adapted from Serfling et al., 2006).

**Regulation of NFAT Activation**

When a T cell is exposed to an antigen-MHC II complex presented by the APCs (antigen presenting cells), the TCR signaling cascade is started. One of the immediate outcome of the TCR signal is the activation of $Ca^{2+}$ / calmodulin-dependent serine phosphatase calcineurin. Usually NFAT is heavily phosphorylated and resides in the cytosol as an inactivated form. Activated calcineurin dephosphorylates NFAT (Fig-1.2) and consequently the dephosphorylated

NFAT (active form) translocates into the nucleus and activates a set of target genes, such as the IL-2, IL-3. IL-4, IL-13, IFN-γ, TNF-α, FasL genes (Jose et. al., 1999).

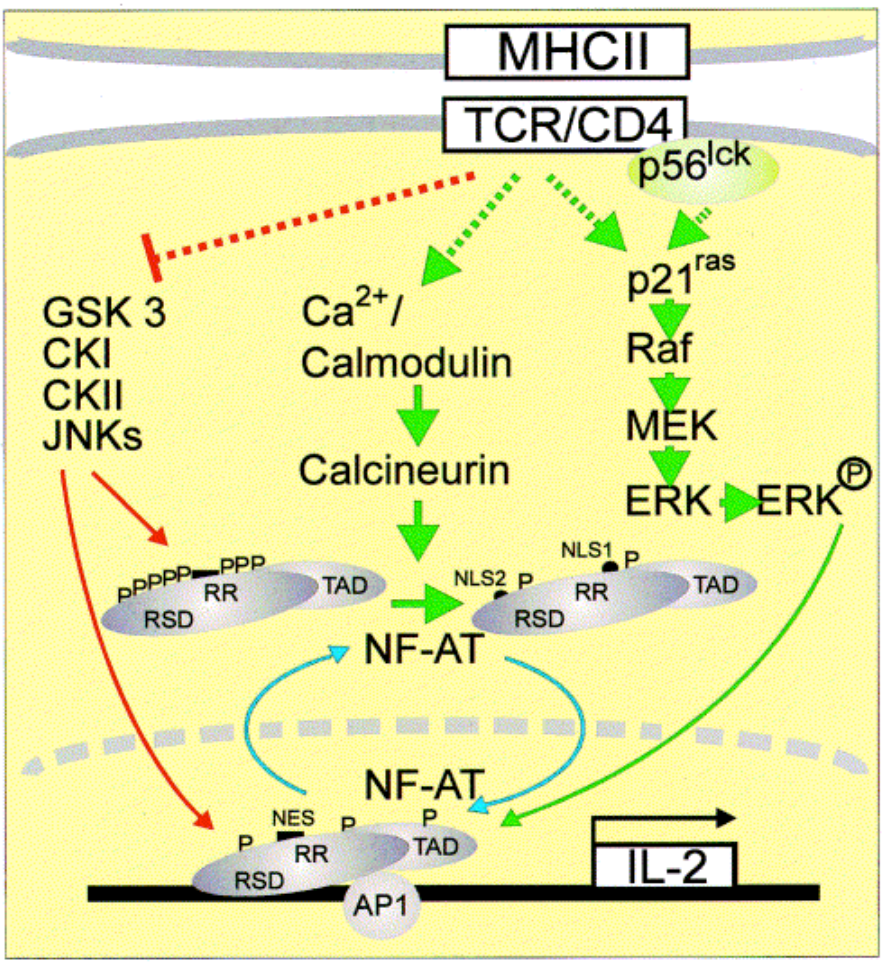



***Fig 1.2*** **Schematic diagram of TCR signaling pathway leading to the activation of NFAT in T cells.** Signalling events from TCR activates calcineurin which dephosphorylates cytosolic inactive NFAT, leading to nuclear translocation of NFAT and subsequently activation of its target gene, for instance the IL-2 (Adapted from Serfling et al., 2000).

**Function of NFAT transcription factor(s)**

NFATc1 and NFATc2 play an important role in effector function of peripheral T cells where these NFATc members are highly expressed. NFATc1 and c2 control the expression of various cytokine genes, such as the IL-2, IL-3, IL-4, IL-5, IFN-γ genes. Additionally, NFAT also targets non-cytokine genes, such as CD40 ligand and CD95 ligand genes (Holtz-Heppelmann et al., 1998). Hence, apart from its effector function regulation, NFATs are also involved in the regulation of cell cycle and apoptosis of T cells. Due to autoregulation, in effector T cell, a massive induction of NFATc1/αA occurs, which rescues the cells from rapid apoptosis (Chuvpilo et al., 2002, Kondo et al., in preparation). This autoregulation phenomena was not observed in case of NFATc1/C, NFATc2 and NFATc3. Different from the short isoform, the long NFATc1 isoforms as well as NFATc2 exhibit a pro-apoptotic phenotype (Chuvpilo et al., 2002). Therefore, different NFAT isoforms and family members appear to play a different role in immunity. NFATc1-deficient mouse embryos die before 14 day of gestation due to defects in their development of cardiac valves and septa (De la Pompa et al., 1998; Ranger et al., 1998a), indicating its role in the heart development. The NFATc2 (-/-) and c3 (-/-) double knockout [DKO]) mice show a disturbed regulation of T cell responses which encompasses massive lymphadenopathy, splenomegaly, and autoaggressive phenomenon (Bopp et al., 2005), suggesting that NFATc2 and c3 in conjunction control T cell proliferation, thereby maintaining T cell homeostatsis.

## *Small Ubquitin- Like Modifier (SUMO)*

### Overview

Post-translational modifications of protein are an important level in gene regulation. Different kinds of post-translational modifications exist in the cell, such as phosphorylation, acetylation, glycosylation, ubiquitinylation that attributes structural variation to the proteins and may alter their function. SUMO is one such kind of post-translational modifier which modify its target protein in a process called sumoylation. SUMO was first discovered in the nuclear transport in mammalian cells as a covalent modification of RanGAP1 (Matunis et al. 1996). SUMO modification targets RanGAP1 to the nuclear pore complex (NPC) where it interacts with Ran binding protein RanBP2 (Mahajan et al., 1997), thereby participating in nuclear import by activating the GTPase activity of the shuttle protein Ran. Since the discovery of SUMOylation, thousands of SUMO modified proteins have been reported till date and the list is ever increasing. Thus, SUMO plays a fundamental role in the regulation of protein function.

**The structure and isoforms of SUMO**

In mammals, four different kinds of SUMO, termed as SUMO-1, SUMO-2, SUMO-3 and SUMO-4, have been identified so far (Dohmen, 2004). Whereas SUMO-2 and SUMO-3 are very similar in sequence, SUMO-1 consisting of about 100 amino acids shows only 50% homology with SUMO-2 and SUMO-3 (Johnson et al., 2004). The SUMO isoforms are diverse in terms of their structure as well as function. SUMO-1 (47% homology with yeast Smt3) is the most predominantly conjugated one under normal cellular condition. On the other hand SUMO2/3 (45% homology with yeast Smt3) preferentially conjugate with their target proteins under cellular stress condition, temperature stress in particular (Saitoh et al., 2000). However, the long isoform of the CAAT/enhancer-binding protein-beta (C/EBPβ-LAT) are modified by SUMO2/3 under normal physiological condition (Eaton et al., 2003). Although the NMR structure of SUMO-1 (Bayer et al., 1998) shows only 18% homology with the X-ray crystallographic

structure of ubiquitin (Fig. 1.3), the two proteins share the same protein folding. Like ubiquitin, SUMO-1 is also comprised of the two conserved C-terminal Gly residues, which are essential for the modification of other proteins in the same spatial arrangement. The most striking difference between SUMO-1 and ubiquitin lies in the extra N-terminal domain of SUMO-1, which is absent in ubiquitin. This extra N-terminal domain of SUMO-1 forms a flexible appendix that protrudes from the core protein. The differential surface charge distribution of SUMO-1 and ubiquitin provides differential specificity for modifying enzymes as well as substrates.

**Sumoylation pathway**

Sumoylation is a process by which a protein is covalently modified by SUMO. The analysis of increasing number of SUMO modified proteins has revealed a consensus sequence where SUMO binds physically. The well-characterized SUMO consensus motif consists of four amino acid sequences, namely Ψ-K-X-E, where Ψ is an aliphatic residue, mainly L (leucine), I (isoleucine) or V (valine), followed by K (lysine) and end with E (glutamic acid). Lysine is the most crucial amino acid in the SUMO consensus motif. Mutation of lysine abolishes the modification of SUMO with its target proteins. It has been shown that the SUMO consensus motif mediates direct interactions with the SUMO-conjugating enzyme Ubc9 and this is essential for SUMO modification (Sampson, 2001).

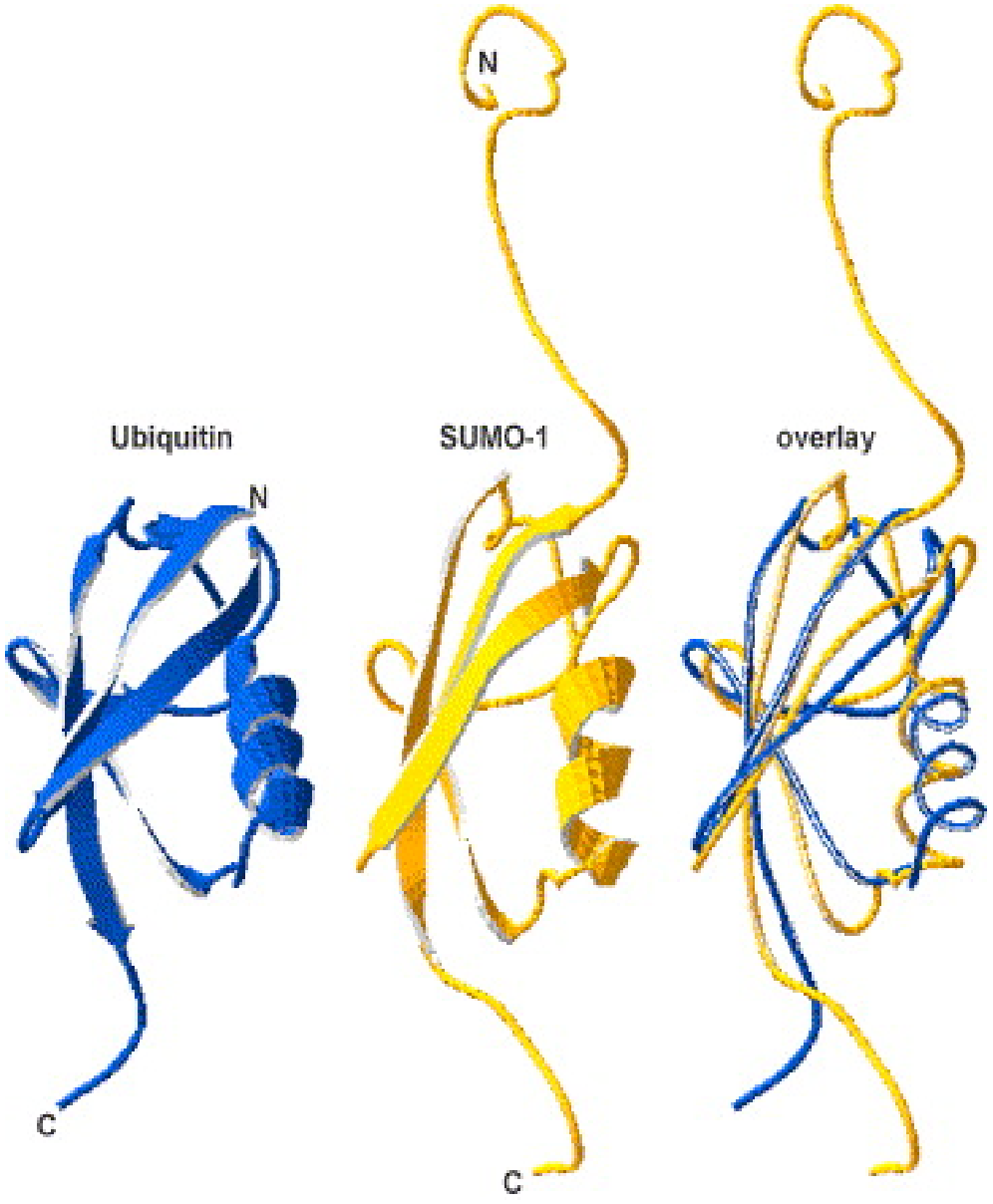
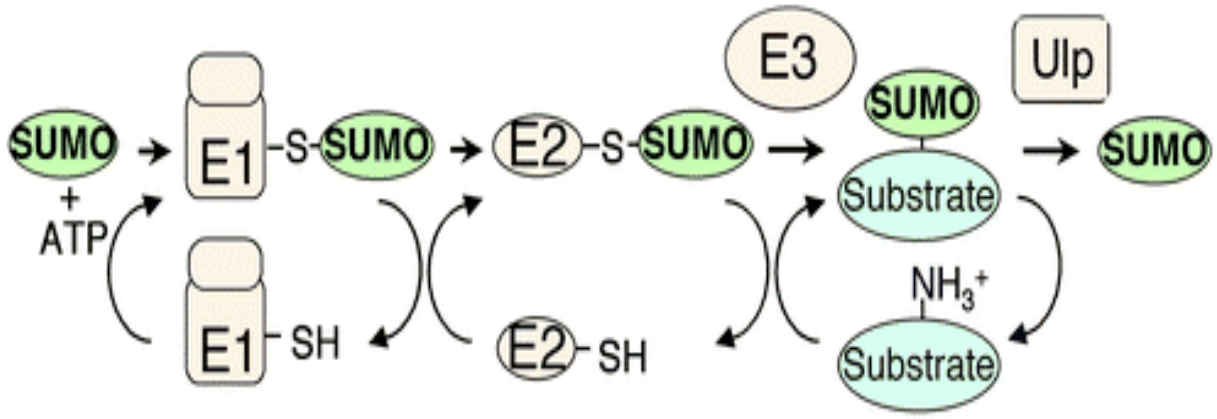



***Fig 1.3*** **X-ray crystallography Structure of Ubiquitin and NMR structure of SUMO-1**. Both proteins show a similar ββαββαβ fold and a C-terminal di-glycine motif. The extra N-terminus is present only in SUMO-1 (Adapted from Dohmen, 2004).




| | E1 | E2 | E3 | SUMO-cleaving protease |
|---|---|---|---|---|
| *S. c.* | Aos1·Uba2 | Ubc9 | Siz1<br>Siz2 (Nfi1) | Ulp1<br>Ulp2 (Smt4) |
| *H. s.*,<br>*M. m.*,<br>*R. n.* | Aos1·Uba2<br>(SAE1,Sua1·SAE2) | Ubc9 | PIAS1 (GuBP)<br>PIAS3 (KChaP)<br>PIASxαβ (ARIP3,Miz1)<br>PIASy<br>RanBP2 (Nup358)<br>Pc2 | SENP1<br>SENP2 (Axam,SuPr-1,SMT3IP2)<br>SENP3 (SMT3IP1)<br>SENP6 (SUSP1) |

***Fig-1.4*** **Schematic representation of the sumoylation pathway,** which consists of maturation and activation of SUMO followed by conjugation to Ubc9 and finally, ligation to its target protein. The list of enzymes involved in this pathway is given below (Adapted from Johnson, 2004).

In the sumoylation pathway (Fig-1.4), a short C-terminus end is cleaved off by the SUMO-activating enzyme E1 as a maturation step (Okuma et al., 1999). In the step, activation occurs via a non-covalently bound SUMO adenylate intermediate followed by formation of a thio-ester bond between SUMO and Uba2 (E1). In the following step activated SUMO is transferred to the SUMO-conjugating enzyme (E2) Ubc9, by a trans-esterification reaction. A non-covalent

interaction occurs between SUMO-Ubc9. Until recently, the physiological relevance of this interaction was not clear. Recent studies established that this non-covalent SUMO-Ubc9 binding promotes SUMO chain formation (Knipscheer et al., 2007). In the last step, the SUMO ligases (E3) mediate the formation of a covalent bond between SUMO and its substrate. While E1 and E2 were sufficient for sumoylation of various SUMO substrates *in vitro*, majority of SUMO substrates require E3 ligase for specific interaction. It is a well-established fact that SUMO isoforms have their overlapping as well as specific substrate preferences, which might bring a different outcome on protein function; for example, MEF2 transcription factor is modified by SUMO-2 and SUMO-3, but not with SUMO1 (Gregoire et al., 2005). Three types of SUMO ligases have been reported so far, 1) the PIAS (protein inhibitor of activated STAT) family of SUMO ligases, 2) RanBP2 and 3) polycomb group protein (Pc2). All of them interact with Ubc9 and enhances sumoylation both *in vivo* and *in vitro* (Dohmen, 2000). SUMO-cleavage enzymes, collectively called SUMO isopeptidases are involved in detaching SUMO- tag from proteins.

## Regulation of sumoylation

The sumoylation pathway (sumoylation/desumoylation) is highly dynamic. The regulation of SUMO conjugation or sumoylation is not well understood. There are no established signaling (both external as well as internal) pathways, which would directly modulate the sumoylation pathway, although the differential and specific choice of SUMO substrates and the time of their modification contribute to the highly selective property of sumoylation pathway. Recent studies have revealed that the phosphorylation status of several substrate proteins, such as of c-jun and PML, affects their sumoylation (Müller et al., 2000, Everett et al., 1999). The effect of phosphorylation on sumoylation appears to be negative. Moreover, Elk-1 transcription factor sumoylation has been shown to be regulated by MAP kinases (Yang et al., 2003). Sumoylation of Elk-1 represses Elk-1-dependent gene expression, in the absence of MAP kinase pathway activation. Upon MAPK-dependent phosphorylation, Elk-1 is de-sumoylated and transcription is activated. However, in case of heat shock transcription factor HSF1, phophorylation induces its sumoylation (Roland et al., 2003). Apart from SUMO modification, lysine residues serve for various other protein modifications, like acetylation, methylation and ubiquitinylation.

Therefore, it is possible that these modifications might regulate each other by simply competing for the same lysine residue. This argument is consistent with the observation that the transcription factor Sp3 contains a lysine that can be acetylated or sumoylated (Sapetschnig et al., 2002). Another line of regulation for SUMO modification occurs by interactions with other macromolecules; for instance, Mdm2 and p53 sumoylation is enhanced by association with the tumor suppressor ARF (Chen et al., 2003). Sumoylation of the base excision repair protein thymine DNA glycosylase (TDG) is stimulated (*in vitro*) both by DNA and its downstream enzyme in the repair pathway (Hardeland et al, 2002). In summary, sumoylation is a highly dynamic and selective process. A fine tuning of this pathway exists both with respect to the choice of substrates as well as to the time of modification.

## Biological functions mediated by sumoylation

**Subnuclear localization**

Sumoylation has profound effects on subcellular as well as subnuclear localization of its target protein. SUMO-modified proteins localize to nuclear bodies, PML nuclear bodies (PML-nbs) in particular. The PML was initially discovered in studies of promyelocytes from patients suffering from acute promyelocytic leukemia (APL), in which a fusion of the PML protein and the retinoic acid receptor α (RARα) occurs as a result of the chromosomal translocation t (15:17) (de The´ et al., 1991, Goddard et al., 1991, Kakizuka, 1991). PML-nbs are also known as PML oncogenic domain (POD), nuclear domain 10 (ND10) or Kremer (Kr) body. There are as many as 5-30 PML-nbs observed per nucleus, ranging from 0.2 to 1 μm (Melnick et al., 1999). PML-nbs are large macromolecular structures harboring different kinds of proteins and loops of chromatin (Boisvert, 2000) (Fig-1.5). Apart from the prime component PML protein, PML-nb is the house for many other proteins, including SUMO-1, Sp100, Daxx, CBP and HDACs (Figure-1.5), which may affect their structure and function. PML Sumoylation and noncovalent binding of PML to sumoylated PML through the SUMO binding motif constitutes the nucleation event for subsequent recruitment of sumoylated proteins and/or proteins containing SUMO binding motifs to the PML-nbs (Shen et al., 2006). Hence, SUMO modification of PML proteins is critical for mature PML-nb formation. PML-nbs are sites for transcriptional hotspots, for activation as well

as repression (Wang et al. 2004; Block et al., 2006). PML-nbs also regulate chromatin-loop architecture and transcription of genes, MHC class I locus for example (Kumar et al., 2007). Apart from the role of sumoylation in PML-nb localization, several studies have also demonstrated that SUMO-modication directs its target protein to pericentric heterochromatin (Arco et al., 2005, Berberich-Siebelt et al., 2006).

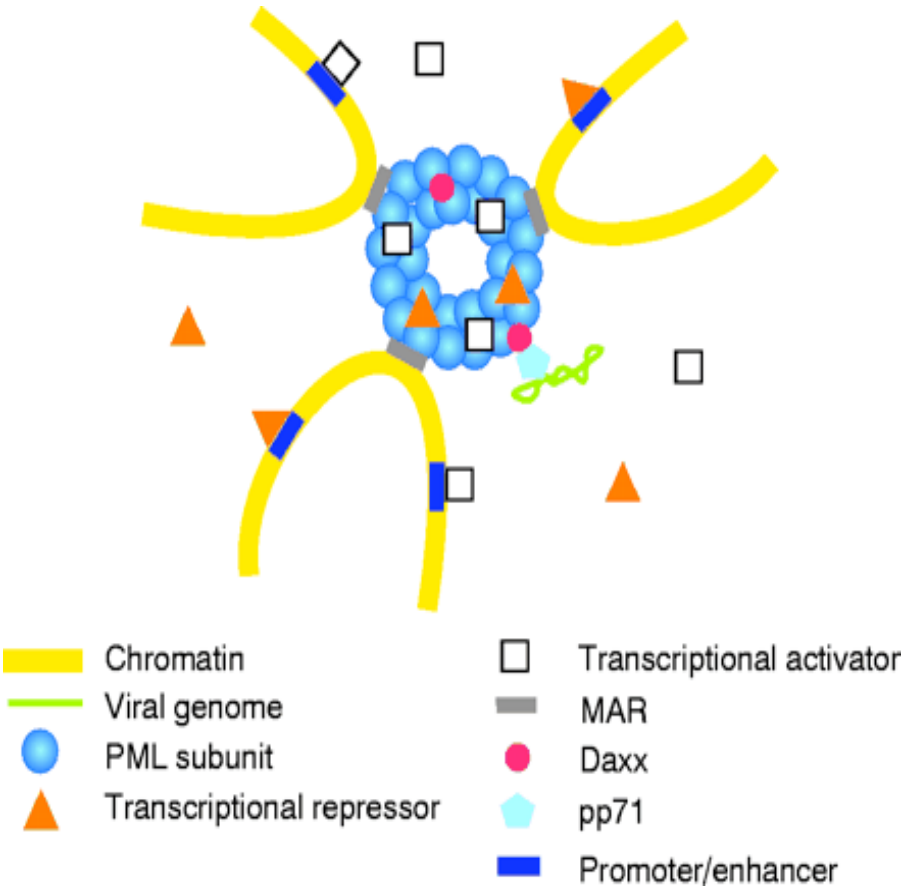



Fig 1.4 Structure of PML-nbs. The basic framework of PML-nbs consists of sumoylated PML monomers. In addition, various kinds of proteins, such as transcription factors, chromatin modifiers etc. are found within the bodies. Chromatin loops are also attached with the periphery of PML-nbs. Upon adenoviral infection, the viral genome localizes to the surface of PML-nbs (Adapted from Ching et al., 2005).

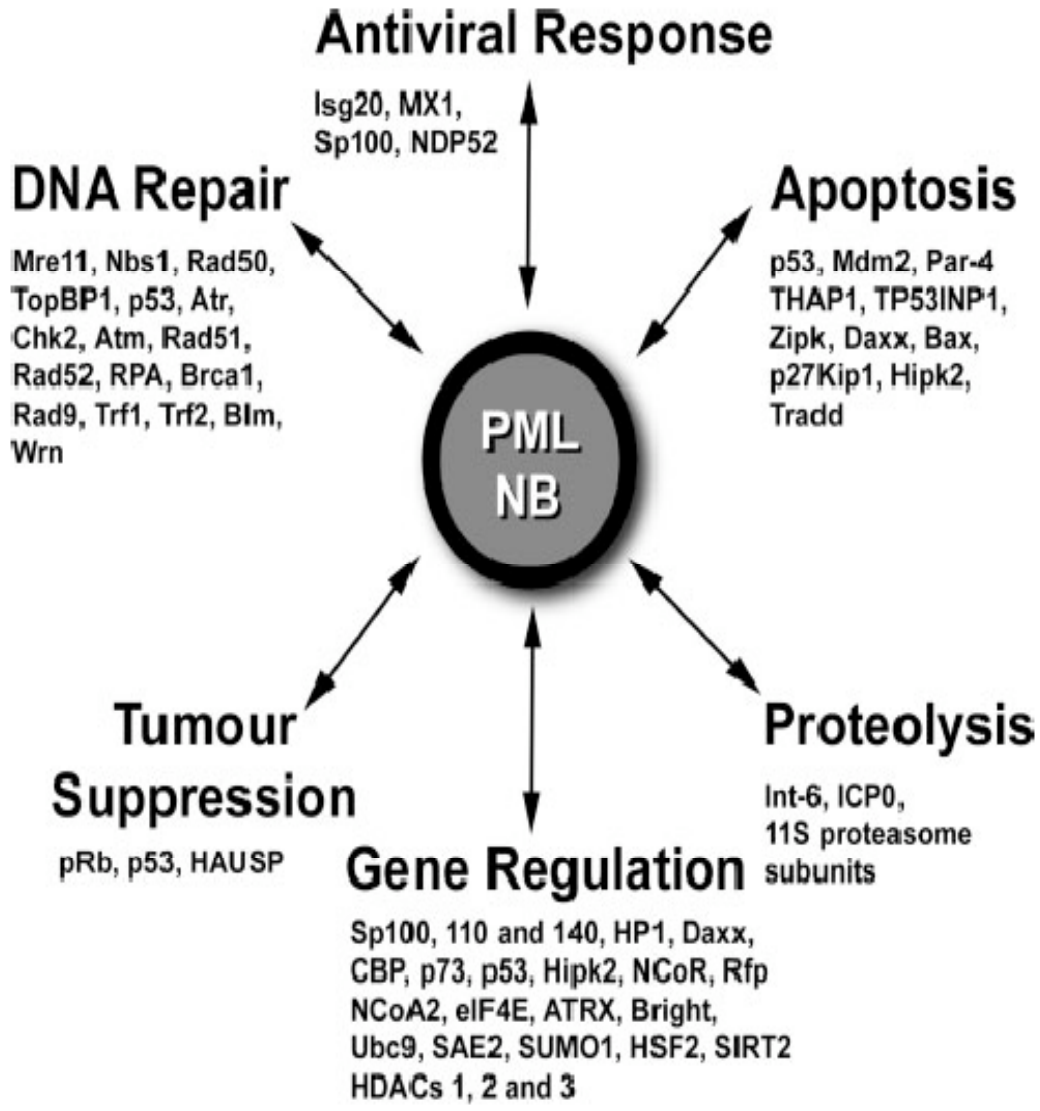



**Fig 1.6 Schematic representation showing a detailed account** for various kinds of proteins observed in the PML-nbs, and their possible role in different biological functions (Adapted from Dellaire et.al., 2004).

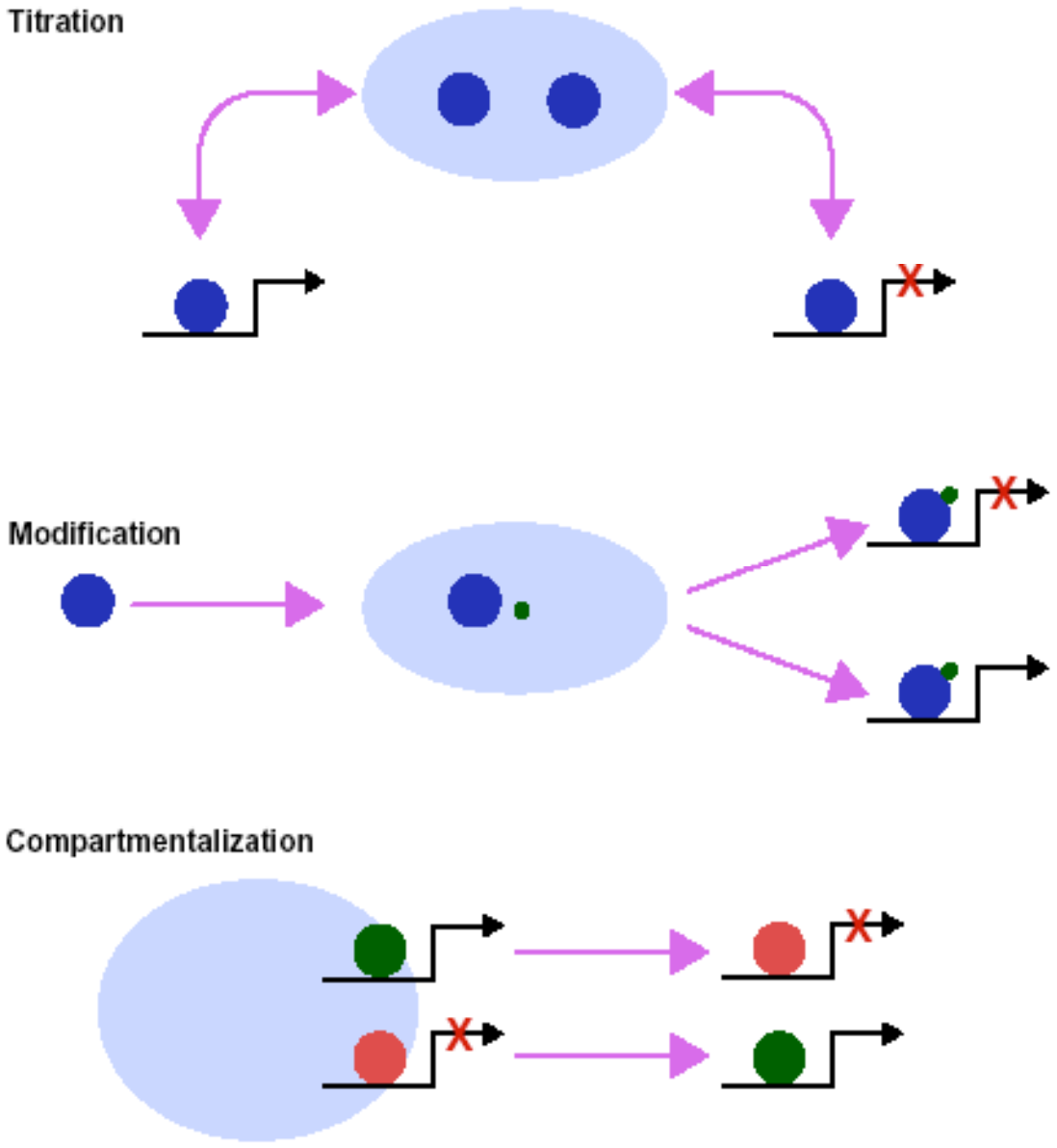



**Fig 1.7 Model showing transcriptional regulation by nuclear-bodies**. The nuclear body such as PML-nb may regulate transcription of various kinds of transcription factor by either sequestration, posttranslational modification followed by compartmentalization. (Adapted from Zong et al., 2000)

**Chromatin structure and function**

SUMOylation plays an important role in higher-order chromatin structure and in chromosome segregation (Watt, 2007), although the molecular basis of these effects are largely unknown. In fission yeast, SUMO modification has been shown to be involved in the maintenance of heterochromatin stability (Shin, 2005). Moreover, a mutant in the *D. melanogaster dpias* gene causes a loss of heterochromatin-induced transcriptional silencing (position effect variegation) of adjacent loci (Johnson, 2004). In addition, SUMO localizes in an area surrounding the kinetochore in mammalian cells, and a number of proteins associate with both centromeres and PML-nbs, suggesting a common, SUMO-related mechanism (Pluta, 1998). Involvement of SUMO modification has been shown in MBD1- and MCAF1-mediated heterochromatin formation (Uchimura et al., 2006). Taken together, these studies indicate an important role of sumoylation in the modulation of chromatin organization.

**Regulation of transcription**

Transcription modulators are the most predominant target proteins of SUMO; therefore, sumoylation plays a pivotal role in the regulation of transcription. Sumoylation regulates transcription of its target proteins either by altering its promoter occupancy or by sequestration into PML-nbs (Seeler et al., 2003). While sumoylation of LRH-1 prevents its binding with its target gene promoter (Chalkiadaki et al., 2005), LEF1 transcriptional activity was repressed upon sumoylation by sequestration it into PML-nbs (Sachdev et al., 2001). In general, Sumoylation has been shown to bring repressive effects on the transcription factor activity. Recently, the ongoing research in many laboratories have focused on trying to understand the mechanism of transcriptional repression by SUMO. Sumoylation is shown to promote interaction of its target protein with transcriptional repressors, HDAC2 in particular (Yang et al., 2004). Furthermore, HDAC6 ‘knockdown’ by si-RNAs relieves SUMO-dependent transcriptional repression by p300. This observation was in line with a model in which SUMO terminates transcription through recruitment of HDAC6 (Girdwood et al., 2003) Although sumoylation of most transcription factors results in transcriptional repression, SUMO seems to have positive effects on the

transcriptional activation by the heat shock factors HSF1 and HSF2 (Hietakangas., 2003).

**Regulation of signal transduction pathways**

An immune response against inflammation includes activation of the NF-κB pathway, where degradation of IκB leads to nuclear translocation of NF-κB and activation of NF-κB target genes. SUMO conjugation of IκB counteracts its ubiquitinylation and thereby leads to inhibition of IκB degradation, thereby inhibiting NFκB mediated gene expression (Desterro et al., 1998). Furthermore, recent studies established evidence that the proinflammatory stimuli activate the IKKα-mediated sumoylation-dependent phosphorylation of PIAS1 for the immediate repression of inflammatory gene activation (Liu et al., 2007). Thus, sumoylation restricts the inflammatory response. Interestingly, sumoylation has an opposite effect on the orthologous pathway in *Drosophila*, where the nuclear translocation of NF-κB ortholog Dorsal is promoted by sumoylation (Johnson, 2004). In *Dictyostelium,* sumoylation controls chemotaxis and aggregation in response to extracellular cAMP, by directly modulating MAP kinase pathways (Sobko et al., 2002).

**Involvement of SUMO in diseases**

Recent studies point to the role of SUMO modification in some important pathological conditions such as in neurodegenerative diseases. SUMO regulates the generation of amyloid β-peptide (Aβ) from amyloid precursor protein (Li et al., 2003), the mechanism of which is not known. While overexpression of SUMO-3 inhibited the generation of Aβ, SUMO-3-K11R (the lysine residue which is involved in the formation of the polySUMO chain is mutated to arginine) overexpression had an opposite effect suggesting that monosumoylation enhances and polysumolyation inhibits Aβ formation (Li et al., 2003). Additionally, SUMO modification has been shown to be involved in the regulation of neurodegenerative diseases caused by polyglutamine (polyQ) repeat expansion, such as in Huntington´s disease (HD) and spinocerebellar ataxias (SCA) (Steffan et al., 2004, Ueda et al., 2002). Furthermore, an increased accumulation of sumoylated protein(s) was observed in the neurons in a transgenic mouse expressing mutant ataxin-1 in the brain (Terashima et al., 2002). In the *Drosophila* model of HD,

it is shown that the sumoylated form of Huntingtin (Htt) is stabilized, to be less likely to aggregate and has an increased capacity to repress transcription, suggesting that sumoylation increases the accumulation of soluble Htt oligomers which mediate neurodegeneration via transcriptional repression (Steffan et al., 2004).

**Miscellaneous functions**

Apart from the above mentioned roles of SUMO, it also plays a significant role in a variety of other biological processes, such as in DNA repair (Hardeland et al., 2002), cell cycle regulation (Li et al., 1999), nuclear transport (Mahajan et al., 1997) etc.

## Hypothesis and objectives of the project

The transcription factor NFATc1 is expressed in short (c1/A) and long (c1/C) isoforms. As described earlier, that NFATc1/C contains an extra C-termial peptide of 246 amino acids. This extra C-terminal part is absent in c1/A. We were interested in studying the specific functions of NFATc1 isoforms and how the functional regulation of these two isoform is achieved. We argued that the extra C-terminus of the long isoform might serve as a platform for different unknown protein interactions, which might regulate NFATc1 differentially and provides a functional specificity between the short and the long isoform. To test this idea, a yeast two hybrid screen was performed by others in our laboratory, where the extra C-terminus part of the long NFATc1/C isoform was used as a bait and for screening a human spleen cDNA library. At the end of this screening, the most predominant interactions detected were with protein regulators involved in sumoylation pathway, such as Ubc9, PIAS1 and Ranbp9. Ubc9 is the only SUMO conjugating enzyme, and PIAS1and Ranbp9 are SUMO ligases. In the yeast two hybrid screen, Ubc9 was precipitated several times indicating a possible strong interaction of Ubc9 with the long isoform of NFATc1. From this preliminary observation we further hypothesized that the long isofom might be sumoylated *in vivo* and the function of long isofom, but not of short isoform, is regulated by sumoylation. To test this hypothesis, which might unravel an additional regulatory mechanism of NFATc1 function, we asked the specific questions below:

1) **Is the long isoform of NFATc1 (c1/C) sumoylated *in vivo*?**
2) **Are there any functional consequences of sumoylation on NFATc1 function?**
3) **How does SUMO-modification regulate NFATc1 function?**

# 2. MATERIALS AND METHODS

## *Materials*

### Mice

$C57^{b6}$ mice were used in this study were either 6 to 8 weeks old; mice were bred in the animal facility at the Institute of Pathology under pathogen free conditions. Institutional guidelines were followed in handling mice throughout the course of the study.

### Cell line

1. 293 HEK
2. EL-4 (Mouse thymoma cell line)
3. A3.01 (Human T cell line)

### Chemicals and reagents

| **Reagents** | **Source** |
|---|---|
| PMA | Calbiochem |
| Ionomycine | Calbiochem |
| Dimethyl Sulfoxoide (DMSO) | Sigma |
| HEPES | Sigma |
| Ethylene glycol tetraacetic acid (EGTA) | Sigma |
| Ethylene diamine tetraacetic acid (EDTA) | Sigma |
| Potassium chloride | Sigma |
| Sodium citrate | Sigma |
| Saponin | Sigma |
| Glycine | Roth |

| | |
|---|---|
| Tris | Roth |
| Boric acid | Roth |
| Magnesium chloride | Roth |
| Sodium chloride | Roth |
| Sodium azide | Sigma |
| Bovine serum albumin (BSA) | Sigma |
| Agarose | Applichem |
| Polyacrylamide | Applichem |
| Sodiumdodecyl sulfate (SDS) | Roth |
| Ammonium persulfate (APS) | Sigma |
| TEMED | Sigma |
| Ethidium bromide | Roth |
| Bromophenol blue | Roth |
| Acetone | Roth |
| Methanol | Roth |
| Ethanol | Roth |
| Chloroform | Roth |
| Isopropanol | Roth |
| Formaldehyde | Roth |
| Formamide | Sigma |
| Cell strainers | Hartenstein |
| Trypan blue | Sigma |
| Proteinase K | SIGMA-Aldrich |
| Complete Protease inhibitor | Roche |
| Gene Ruler™ 100bp ladder | MBI Fermentas |
| ECL chemiluminescence kit | Pierce |
| Human IL-2 ELISA kit | BD Pharmingen |
| Protein G sepharose | Upstate |
| Protein A sepharose | Upstate |

| | |
|---|---|
| NP-40 | Sigma |
| Bradford's Reagent | BioRad |
| Nitrocellulose membrane | Hartenstein |
| Whatman filter paper | Hartenstein |
| 96 well cell culture plates | Cellstar |
| 6 & 24 well cell culture plates | Cellstar |
| 12 well cell culture plates | TPP, Switzerland |
| 6 mm Petri dishes | Cellstar |
| T-25, T-75 & T-250 tissue culture flasks | Nunc |
| Cuvettes | Hartenstein |

## Antibodies

### For T cell stimulation :

| | |
|---|---|
| αCD3 (145.2C11) | BD Pharmingen |
| αCD28 (37.51) | BD Pharmingen. |

### For immunoprecipitation and western blot analysis:

| | |
|---|---|
| Mouse anti-mouse NFATc1 | Alexis |
| Rabbit anti-human NFATc1 (AB1-205) | ImmunoGlobe |
| Goat anti-mouse HRP | Sigma |
| Rabbit anti-goat HRP | Sigma |
| Mouse-anti-FLAG M2 | Sigma |
| Mouse-anti-HA | Roche |
| Mouse-SUMO-1 | Santa Cruz Biotech |
| Mouse-anti-c-Myc | Santa Cruz Biotech |
| Mouse-anti-ERα | Santa Cruz Biotech |

## For confocal microscopy:

| | |
|---|---|
| Rabbit anti-NFATc1 | ImmunoGlobe |
| Mouse-anti-HA | Roche |
| Mouse-SUMO-1 | Santa Cruz Biotech |
| Goat-PML | Santa Cruz Biotech |
| Rabbit anti-H3K9 (Trimethylated) | Upstate |
| Mouse anti-RNA polymerase II | Upstate |
| Donkey anti-Rabbit Cy3 | Dianova |
| Donkey anti-Rabbit Cy5 | Dianova |
| Donkey anti-Mouse Cy3 | Dianova |
| Donkey anti-Mouse Cy5 | Dianova |
| Donkey anti-goat Cy2 | Dianova |
| DAPI | Dianova |
| Fluoromount G | Dianova |

## PCR Primers for Chromatin Immunoprecipitation (ChIP) :

hIL-2 promoter

F 5'- CTT GCT CTT GTT CAC CA- 3'

R: 5'- CTG TAC ATT GTG GCA GGA-3')

h β-actin promoter

F: 5'- CCT CCT CCT CTT CCT CA -3'

R: 5'- CGA GCC ATA AAA GGC AA -3

## PCR reagents:

| | |
|---|---|
| dNTPs | MBI Fermentas |
| $MgCl_2$ | MBI Fermentas |

| | |
|---|---|
| 10x Buffer | MBI Fermentas |
| Taq Polymerase | MBI Fermentas |

## *Mediums and buffers*

## Cell culture:

**BSS (Balanced Salt Solution):**

1.07 g BES (N,Nbis[2-hydroxy-ethyl]-2-aminoethanesulfonic acid)

1.6 g NaCl

0.027 g $Na_2HPO_4$

in 100 ml dd$H_20$ pH 6.96

+ 0.1% BSA (Bovine Serum Albumin)

**PBS (Phosphate buffered saline):**

| | |
|---|---|
| 8 g | NaCl |
| 0.2 g | KCl |
| 1.44 g | $Na_2HPO_4$ |
| 0.24 g | $KH_2PO_4$ |

Dissolve in 800 ml $dH_2O$, adjust pH to 7.4 with HCl

Volume adjust to 1 L, autoclave and store at RT

| | |
|---|---|
| $RPMI^+$ 1640 Medium: | Invitrogen, Karlsruhe |
| +L-Glutamin (0.07%) | GIBCO |
| +Na-Pyruvat (1 mM) | GIBCO |
| +β-Mercaptoethanol (0.05 mM) | GIBCO |
| +MEM (non essential amino acids) (1%) | GIBCO |
| + Penicillin (100 U/ml), | Grunthal |
| + Streptomycin (100 U/ml) | FatolGmbH |
| + FCS (Fetal calf serum) (1-10%) | GIBC0 |

(heat inactivated at 56°C for 45 min)

## Agarose gel electrophoresis:

**TBE Buffer (1x):**

10.8 g Tris

5.5 g Boric acid

0.37 g EDTA

add 1 l $H_2O$

**DNA Loading Buffer:**

0.25% Bromophenol blue

0.25% Xylene Cyanol FF

30% Glycerol in water

store at 4°C

*1% Agarose Gel: (150 ml)*

1.5 g agarose

150 ml 1x TBE buffer

boil

add 4 µl Ethidium bromide (10 mg/ml)

**IL-2 ELISA**

*Coating buffer (0.1 M Sodium Carbonate, pH 9.5)*

8.40 g $NaHCO_3$

3.56 g $Na_2CO_3$

Volume adjusted to 1L with $dH_2O$

Assay diluent
PBS with 10% Fetal Bovine Serum, pH 7.0

Wash buffer

PBS with 0.05% Tween-20

Substrate solution
Tetramethylbenzidine (TMB)

Hydrogen peroxide ($H_2O_2$)

*Stop solution*

2 N $H_2SO_4$

**Cell counting**

*Trypan Blue Solution:*

0.4% (w/v) Trypan Blue in PBS

Dilute 1:10 in PBS for live cell counting

**Cell extract preparation**

*Whole Cell Extract (WCE) Buffer:*

5mM Tris HCL, pH7.4

150mM NaCl

1mM EDTA

1% Triton X-100

Protease inhibitor cocktail was added immediately before use.

*Cell lysis buffer for IP:*

5mM Tris HCL, pH7.4

150mM NaCl

1mM EDTA

1% Triton X-100

Protease inhibitor cocktail was added immediately before use.

*(10X) Wash buffer for IP:*

0.5 M Tris Hcl, pH 7.4, 1.5M NaCl

**SDS-PAGE**

Composition of SDS-Polyacrylamide gels
Resolving Gel:
Component volumes (ml) per 10 ml gel mix

| | 8% | 12% |
|---|---|---|
| $H_2O$ | 4.6 ml | 3.3 ml |
| 30% acrylamide mix | 2.7 ml | 4.0 ml |
| 1.5 M Tris (pH 8.8) | 2.5 ml | 2.5 ml |
| 10% SDS | 0.1 ml | 0.1 ml |
| 10% ammonium persulfate | 0.1 ml | 0.1 ml |
| TEMED | 0.006 ml | 0.004 ml |

*Stacking Gel:*

Component volumes (ml) per 3 ml gel mix

| | |
|---|---|
| $H_2O$ | 2.1 ml |
| 30% acrylamide mix | 0.5 ml |
| 1.0 M Tris (pH 6.8) | 0.38 ml |
| 10% SDS | 0.03 ml |
| 10% ammonium persulfate | 0.03 ml |
| TEMED | 0.003 ml |

*Protein Loading Buffer (1X SDS Gel Loading Buffer):*

50 mM Tris-Cl (pH 6.8)

100 mM Dithiothreitol

2% SDS

0.1% Bromophenol Blue

10% Glycerol

store at -20°C

*Gel Running Buffer: (10X)*

144.13 g Glycine

30.3 g Tris

100 ml 10% SDS

volume adjusted to 1l with $dH_2O$

store at RT

*Protein Transfer Buffer: (10X)*

145 g Glycine

29 g Tris

volume adjusted to 1L with $dH_2O$

store at RT

dilute 10X transfer buffer: methanol: $dH_2O$ in 1:2:7 ratio before use

**Blocking Solution:**

5% (w/v) nonfat dried milk in PBS/0.1% Tween

**RNA analysis:**

*DEPC $H_2O$*

0.1% DEPC in $dH_2O$

stirr until DEPC is completely mixed in $H_2O$

Autoclave and store at RT

*MOPS Buffer: (10X)*

0.4 M MOPS

0.1 M NaOAc

0.01 M EDTA

pH 7, autoclave and store in dark at RT

*Formaldehyde Buffer:*

15% Formaldehyde

50% Formamide

10% 10X MOPS buffer

*RNA Loading Buffer:*

0.5% Xylene cyanol

0.5% Bromophenol blue

10 mM EDTA

50% Glycerol

30% Formamide

*Fomaldehyde-Agarose gel electrophoresis:*

For 150 ml of 1.2% gel

1.8 g Agarose

15 ml 10X MOPS buffer

98 ml $dH_2O$

boil and then add 27 ml formaldehyde in the fume hood

**Luciferase assay**

*Luciferase harvesting buffer*
1.5 M Tris, pH 7.8

1 M MES

Triton X-100

DTT (1 M stock) was added just before use (50µl DTT for 10ml buffer volume)

*Luciferase assay buffer*
1.5 M Tris, pH 7.8
1 M MES
1 M $Mg(C_2H_3O_2)_2$. $4H_2O$
A pinch of ATP was added just before use.

*Luciferin solution*

1 M $KHPO_4$, pH7.4
The sterile solution aliquot was kept at -20°C

**For Immunofluorescence**

*Fixing solution*

4% formaldehyde in PBS

*Fixing solution*

0.2% Triton X-100 in PBS

*Blocking solution*

Normal donkey serum in PBS(1:20)

**For ChIP**

*Swelling buffer*
25mM HEPES, pH 7.8
10mM KCl
1.5mM $MgCl_2$
0.1% NP40

Protease inhibitor cocktail was used just before using.

*Sonification buffer*
50mM HEPES, pH 7.8
140mM NaCl
1mM EDTA, pH8.0
1% Triton X-100
0.1% sodiumdeoxycholate
0.1% SDS
Protease inhibitor cocktail was used just before using.

*High salt buffer*

500mM NaCl in sonification buffer

*LiCl buffer*

20mM Tris, pH 8.0
250mM LiCl
1mM EDTA, pH 8.0
0.5% NP-40
0.5% sodiumdeoxycholate

*Elution buffer*

50mM Tris 8.0
1mM EDTA
1% SDS

*TE buffer*

20mM Tris, pH 8.0
1mM EDTA, pH 8.0

## Instruments and accessories

| | |
|---|---|
| Autoclave | Stiefenhoefer |
| Balance machine | Hartenstein |
| Confocal microscope | Leica TCS SP II |
| $CO_2$ incubator | Heraeus Instruments |
| Cytospin Centrifuge | Shandon |
| Dynal Magnet | Dynal A.S. |
| DNA sequencer 373A | Perkin Elmer |
| ELISA-Reader "ν-max" | Molecular Devices |
| Gel documentation system | Herolab |
| Heating blocks | Hartenstein |
| Luminometer | Berthold |
| Microcentrifuge | Hartenstein |
| PCR machine | MWG Biotech |
| pH meter | Hartenstein |
| Spectrophotometer | Pharmacia Biotech |
| Western blot apparatus | Hoefer |

**DNA Constructs**

Luciferase reporter gene plasmid *pTATAluc+* (Altschmied, Duschl, 1997) containing 293 bp of the proximal murine IL-2 promoter (Serfling et al., 1995), retroviral vectors *pEGZ/MCS* (Kuss et al., 1999) and *pEGZ/MCS-HA*, *pHA-hNFATc1/A* and *pHA-hNFATc1/C* expression constructs were used In addition, NFATc1 isoforms and mutants were ued at their 3' ends with the modified hormone-binding domain of the estrogen receptor as described for a chimeric *c-MycER*$^{TM}$ construct (Littlewood et al., 1995) or accordingly, to enhanced yellow fluorescence protein (EYFP) (bicistronic vector *pIZ-EYFP*, a kind gift of I. Berberich). Also SUMO1 was fused in frame to NFATc1, but to its 5' end, where the two glycines at the C-terminus of SUMO1, recognized by the (de)-sumoylation machinery, were mutated to alanine. The oligos for

the exchange of lysines to arginines within the SUMO-consensus sites of NFATc1 were designed as followed: Arg1 (ΔD) CAGTGGCGCTC**CG**GGTGGAGCCC, Arg2 (ΔA) CGTTCCAATTATA**C G** AACAGAACCCAC and Arg3 (ΔB) CTGTAACGGTC**CG**GCGAGAGCC. *pcFlag-SUMO* and *pcFlag-Ubc9* were provided by H. Hofmann (Hofmann et al., 2000) and the *pcDNA-HDAC–myc-His* constructs by the T. Kouzarides laboratory.

## *Methods*

### Transient transfection, reporter gene activity assay

293T HEK cells were transfected by using the conventional calcium phosphate technique and EL-4 cells by a standard DEAE/dextrane protocol. After 36 hrs of transfection, luciferase activity was measured from the cells that were either left untreated or treated with T/I for overnight. Relative light units were corrected for the transfection efficacy due to total protein concentrations (rLA = 0.5 / $Prot_{conc}$ x aLA). Normalized mean values of at least three independent experiments are depicted in relative light units (RLU) or as fold activation over empty vector control in the cotransfection experiments. Western blots were performed from the same protein extracts to check the equal expression pattern of the exogenously expressed proteins. For immunofluorescence, 293T cells were transfected by superfect method according to the protocol of Quiagen (Valencia, CA) with different HA-NFATc1 and Flag-SUMO-1 constructs. After 48 hrs of transfection, cells were stimulated with 10ng TPA, 1μM ionomycine and 2mM $CaCl_2$.

### Immunoprecipitations and Western blot assays

For IP 107 cells were harvested 36 hrs post transfection and resuspended in 1 ml lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitor cocktail, Roche, and 20 mM N-ethylmaleimide, Sigma-Aldrich). 50 ml were kept as lysate control. Otherwise, the whole cell extract was incubated with 3 μg anti-Flag (M2, Sigma), 3 μg anti-Myc (sc-40, Santa Cruz) or 3 μg of NFATc1 Ab (AB1-205, ImmunoGlobe, Germany) and

40 µl of protein A or G beads (Upstate, USA). The beads were then washed three times with washing buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, protease inhibitor cocktail). For Western blot the precipitates and input control lysates were fractionated by 8 - 12% SDS-PAGE and electroblotted onto nitrocellulose membrane. For detection anti-HA Ab (M-12CA, Roche Diagnostics), anti-Flag Ab (M2, Sigma) or anti-SUMO Ab (sc-5308, Santa Cruz) and a peroxidase-coupled secondary Ab were used with a standard enhanced chemiluminescence system (Pierce, USA).

**Chromatin immunoprecipitation (ChIP)**

$5 \times 10^7$ retrovirally infected A3.01 cells were stimulated with Tm + T/I for 6 hrs. Fixation was performed wit 1% formaldehyde directly in the medium. After 10 min 125mM glycine was added (for 5 min.) to stop fixation. Cells were washed in 40 ml 0.1% BSA/ PBS for 5 mins, 1400 rpm at 4°C. 10ml of swelling buffer was added to swell the cell on ice for 30 min. Cells were lysed with 400 µl 10% NP-40 (final 0.4%) and vortexed for 10 sec. Nuclei were collected by centrifigation for 10 min, 2500 rpm at 4°C. The nuclei were then resuspended in 600 µl of sonification buffer. The nuclei were disrupted by 2 freeze/thaw cycles by vortexing, followed by sonification 3 times for 30 sec in each time with maximum power, 0.5 pulse/sec. Chromatin was precleared with 3 µg of unrelated Ab and 30 µl of protein A beads (50% slurry saturated with salmon sperm DNA), rolling for 2hrs at 4°C. DNA concentration was determined by spectrophotometry O.D. 260nm. For immunoprecipitation, 30 O.D. of chromatin was taken in 300 µl of sonification buffer with 3 µg of Ab (directed against H3-Ac or ERα) and kept for rolling at 4°C overnight. Next day, 30 µl protein A slurry was added for 2hrs at 4°C, followed by washing of beads twice each with 600 µl sonification buffer, high salt buffer, LiCl buffer and TE buffer. Elution of chromatin was performed (twice) by incubating the beads with 250 µl of elution buffer while rotating the tubes at 65°C. To remove formaldehyde crosslinking, 25µl of 5M NaCl was added and kept for overnight incubation at 65°C. In the following day, 2.5 µl proteinase K (10mg/ml) was added and kept for 1 hr at 42°C. DNA was extracted by phenol/chloroform/isoamylalcohol. Purified DNA was dissolved in 25 µl of TE buffer. The DNA

was then used in PCR amplification. In PCR, either for human IL-2 promoter or the beta actin promoter was amplified which gave rise to 493 bp or 193 bp amplified product, respectively.

**Immunofluorescence and Confocal microscopy**

For NFATc1, SUMO-1 and PML tri-colocalization as well as for heterochromatin and RNA pol II colocalization studies, NFATc1-ER-infected A3.01 cells were harvested on slides by cytospins, while transiently transfected 293T HEK cells had been grown on coverslips. Cells were fixed in 4% formaldehyde and permealized with 0.2% triton X-100 followed by blocking in 1:20 times diluted donkey serum for 20 min. For primary Ab staining, rabbit polyclonal NFATc1-NT (1:200), mouse monoclonal HA (1:500), mouse SUMO-1 (1:50), goat PML (1:50, sc-9862, Santa Cruz), rabbit polyclonal anti-trimethyl-Histone H3 (Lys9, 1:500, 07-442, Upstate), and mouse monoclonal pol II (1:500, 05-623, Upstate) antibodies were used. The secondary antibody combinations used were anti-rabbit Cy3 (1:500), anti-mouse Cy5 (1:500) and anti-goat Cy2 (1:100), respectively (Jackson Immunooresearch). The slides were then mounted with Fluoromount-G (Southern Biotechnology Associates) containing DAPI, and images were collected with confocal microscope (Leica TCS SP2 equipment). For endogenous NFATc1 sumoylation, mouse $CD4^+$ and $CD4^+CD25^+$ cells were collected on slides by cytospin (300rpm, 2min) and dried overnight at room temperature. Formaldehyded fixation, permealization and staining were as mentioned above.

**Viral infection, RNA purification and RNase protection assay.**

Viral infection was carried out as described previously (Berberich-Siebelt et al., 2000). RNA was purified using Trizol reagent (Invitrogen), and 5 µg RNA was analysed by RNase protection assays (RPAs) performed according to Pharmingen`s Riboquant$^{TM}$ protocol. As multi-probe template set, the hCK1 and mCK1 sets were used. ELISAs were performed for detection of IL-2 secretion using mAbs according to the manufacturer's instructions (BD Biosciences, USA).

**ELISA (Enzyme Linked Immunosorbent Assay)**

A3.01 cells infected with different NFATc1 containing retroviruses were taken from ELISA to check the effect of NFATc1 sumoylation on endogenous IL-2 production.

Day 1: The purified anti-cytokine antibody was diluted to 1-4 μg/ml in ELISA coating buffer, 100 μl of diluted antibody was added to each well of 99 well plates and incubated overnight at 4°C.

Day 2: The plate was washed with 1X ELISA washing buffer followed by blocking of non-specific binding by 200 μl of ELISA blocking buffer. The plate was kept at room temperature (RT) for 2-3 hrs followed by washing 1X with ELISA washing buffer. Standard and samples were diluted in ELISA sample dilution buffer, 100 μl in each well for overnight at 4°C.

Day3: The plate was washed at RT four times with ELISA wash buffer. 1μg/ml biotinylated anti-hIL-2 antibody was added in each well and incubated for 1-2 hr at RT. 11μl streptavidin and 11μl AP (alkaline phosphatase) solutions were mixed in 300 μl of ELISA sample buffer. 100 μl of the diluted sterptavidin – AP complex solution was added to each well and incubated 1 hr. After incubation, the plate was washed for eight times with ELISA washing buffer at RT. 100 μl ELISA color development solution was added to each well and the color was measured at 405nm O.D (490nm as reference). The outcome of cytokine production was calculated against the statndard curve in microsoft EXCEL program.

# 3. RESULTS

## *NFATc1/C interacts with the E3-ligase Ubc9*

NFATc1 is expressed in six isoforms. Regarding the N-terminus, these isoforms were designated as NFATc1/a and NFATc1/b. In the context of the C-terminus; they were designated as c1/A, c1/B and c1/C. The long isoforms c1/aC and bC harbor an extra C-terminal peptide of 246 amino acids containing an additional transactivation domain. Therefore, it was likely that this extra C-terminal peptide serves as a platform for protein interactions and, thereby gives functional specificity between the NFATc1 isoforms. In a yeast two hybrid screen using this extra C-terminal peptide as a bait and human spleen cDNA library as prey, Ubc9 (37x) and PIAS1 (4x) were detected as the most predominant NFATc1/C-specific interaction partners. Ubc9 is the only known SUMO-conjugating enzyme, whereas PIAS1 acts as a substrate-specific SUMO ligase. Within NFATc1/C (c1/C) three SUMO consensus sites were identified at the position of amino acid (aa) 349, 702 and 914, while the shortest isoforms of NFATc1/A (c1/A) harbour only the site at aa 349 (Fig. 3.1). The N-terminal a or b-peptides are free of SUMO-consensus sites. In silico analysis predicted strong sumoylation for the C-terminal SUMO consensus sites at lysine 702 and 914, but relatively weak for the general SUMO site lysine 349 (Fig. 3.2).

In so-called delta- (D)SUMO mutants we encoded arginines instead of those sumoylatable lysines by site-directed mutagenesis and named the constructs as follows (Fig. 3.1); K702R is NFATc1/CΔA (ΔA), K914R is ΔB and, if both lysines on the C-terminus are mutated ΔC, i. e. K702, 914R. ΔD refers to the general SUMO site (K349R). In the case of K349/702/914R the designation is ΔE. The mutation within the short isoform is named c1/AΔD. For better comparison, all constructs contain the N-terminal a-peptide.

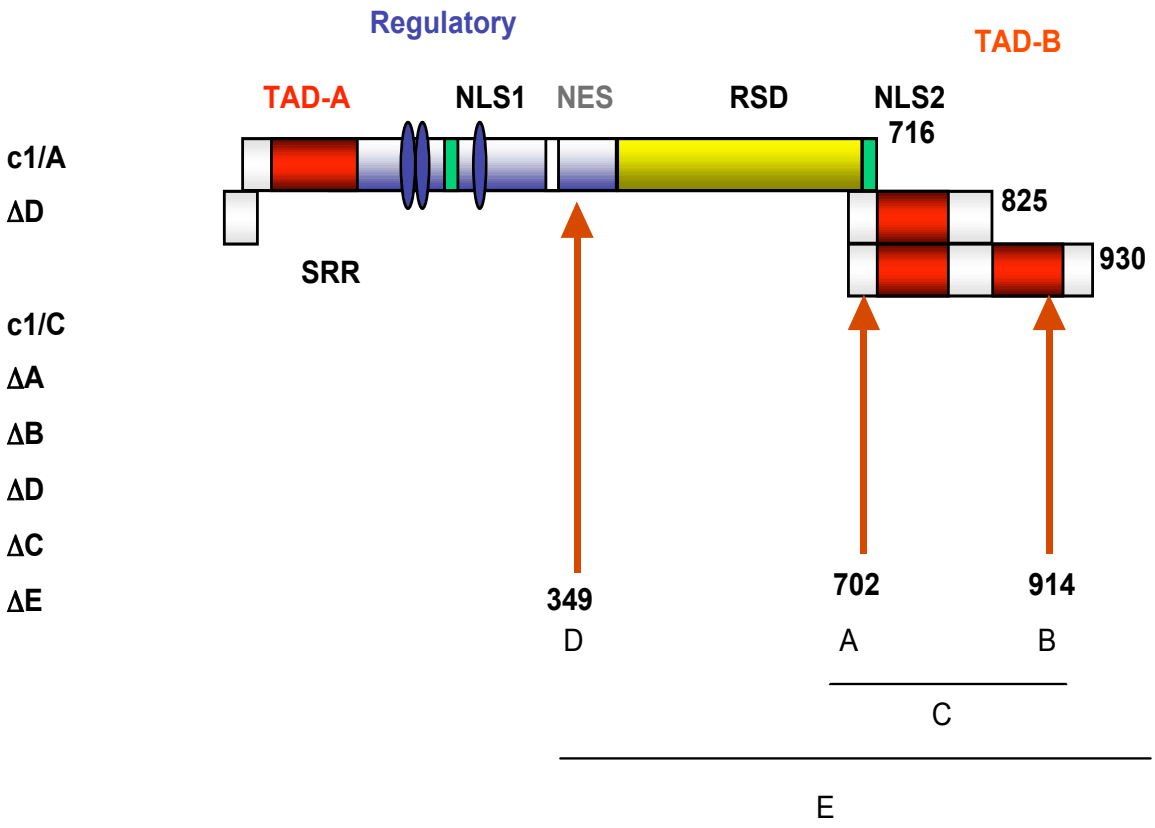



**Figure 3.1 Generation of SUMO consensus motif mutant NFATc1 by site directed mutagenesis.** Schematic representation of NFATc1 isoforms and the SUMO consensus motifs (**Ψ-K-x-E**; Ψ= Isoleucin/Leucin/Valin) are shown by arrows. The short isoform has only one SUMO site designated as the D site; the long isoform harboring the extra C-terminus ternimus contains the A and B sites. Lysine to arginine mutations of 702 and 914 SUMO sites are represented by ΔA and ΔB, respectively. Mutation of both C-terminus lysines is called as ΔC mutation. The general SUMO site mutation on 349 lysine is ΔD mutation. The mutant containing all three SUMO site mutations is designated as ΔE mutant.

The authenticity of the yeast two hybrid screening was verified by transfecting 293T HEK cells with HA-tagged NFATc1 and Flag-tagged Ubc9 encoding constructs followed by immunoprecipitation (IP). c1/C was precipitated with Ubc9, but not in the presence of the empty

vector pcFlag which specifies the c1/C and Ubc9 interaction (Fig. 3.3, left panel). At least two precipitated bands were detected in the IP. Interestingly, when ΔE (which is the mutant for all three SUMO sites) was used no bands at all appeared indicating that the SUMO consensus sites on c1/C are necessary for proper Ubc9 interaction (Fig. 3.3, right panel).

| No | Pos. | Group | Score |
|---|---|---|---|
| 1 | K702 | ANVPI **IKTE** PTDDY | 0.94 |
| 2 | K914 | PIPVT **VKRE** PEELD | 0.93 |
| 3 | K349 | PPSVA **LKVE** PVGED | 0.91 |
| | | Sumoylation motif: Ψ**-K-x-E**; Ψ - Isoleucin/Leucin/Valin | |

**Figure 3.2. *In silico* prediction of SUMO consensus motifs in NFATc1.** An amino acid sequence of human NFATc1 long isoform protein was analyzed by SUMOPLOT software, which search for the SUMO consensus sites on any given protein. SUMO prediction of NFATc1 revealed that the C-termius lysines (702 and 914) are having comparatively higher probability of sumoylation than that of the general SUMO site (349).

### *NFATc1/C is sumoylated* in vivo

We next sought to check if c1/C is indeed ligated to SUMO. Co-transfections of HA-tagged c1/C and Flag-SUMO1 were done into 293T HEK cells. After IP, several (3 - 4) SUMO-modified c1/C bands were observed (Fig. 3.4). In order to see if the predicted SUMO-sites are of importance and if the (de-) phosphorylation state of NFATc1/C is influencing sumoylation, 293T HEK cells were transfected either with c1/C or c1/CΔE plasmids along with Flag-SUMO1 or pcFlag. The cells were left untreated or activated by T/I which mock TCR signalling, thereby leading to dephophorylation and nuclear translocation of NFATs. Although, SUMO-modified c1/C bands were detected in both

untreated and T/I-treated cells, the bands were enriched upon T/I-treatment, indicating dephosphorylation and/or nuclear translocation as support for efficient sumoylation (Fig. 3.5, lanes 1+2).

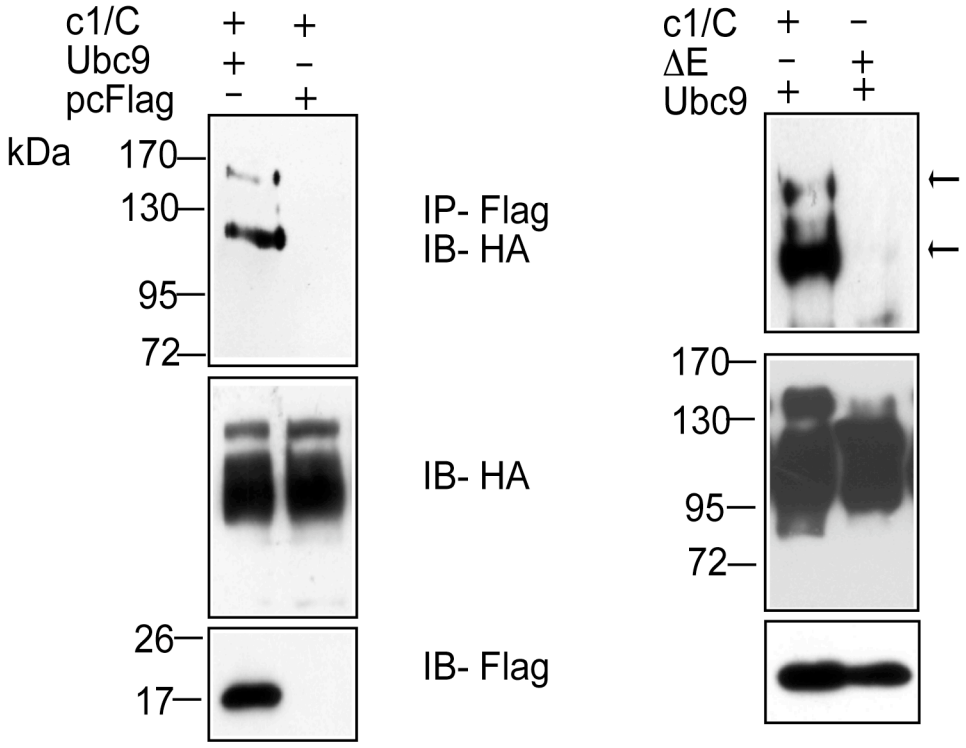



**Figure 3.3 NFATc1/C physically interacts with Ubc9 *in vivo*.** Vector encoding HA-tagged c1/C or ΔE mutant was transfected into 293T HEK cells either with Flag-tagged Ubc9 or pcFlag. After 48 hrs of transfection lysate was prepared having NEM in the lysis buffer. Flag antibody was used for immuoprecipitation followed by western blot detection by HA antibody. Arrows show the NFATc1-Ubc9 interaction.

More importantly, no SUMO-modified band was detected in ΔE, neither in the presence or absence of T/I (Fig. 2B, lanes 3+4). To exclude false precipitation the reverse experiment was performed with precipitation of c1/C by anti-NFATc1 and western blot detection by anti-SUMO1. Bands of identical size demonstrated the specificity of SUMO1-modified c1/C (Fig. 3.6 A). Since SENPs

(SUMO-specific proteases) render sumoylation very transient, the necessity of N-ethyl-maleimide (NEM, a SENP inhibitor) within the lysis buffer was checked. Accordingly, sumoylated c1/C bands could only be observed when NEM was included in the lysis buffer, which points to the highly dynamic and transient nature of sumo modification (Fig. 3.6 B).

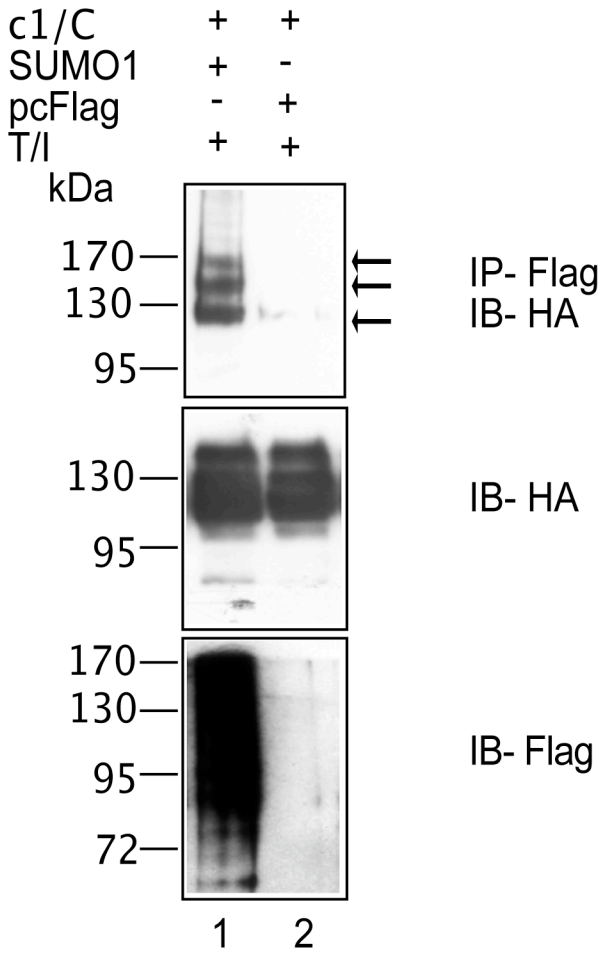



**Figure 3.4 NFATc1/C is sumoylated *in vivo***. Vector encoding HA-tagged c1/C was transfected in 293T HEK cells either with Flag-tagged SUMO1 or pcFlag. NEM was added with lysis buffer. Flag antibody was used for immunoprecipitation followed by western blot detection by HA antibody. Arrows show the SUMO-modified NFATc1 protein bands.

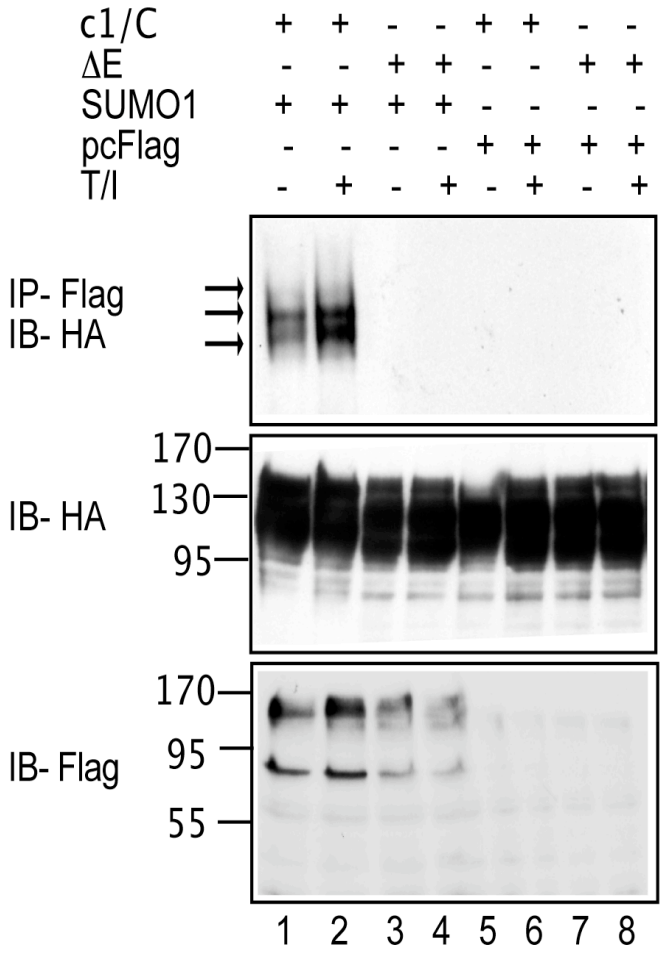



**Fig 3.5 Dephosphorylation of NFATc1 is necessary for its efficient sumoylation.** Either vector encoding HA-tagged c1/C or ΔE mutant was transfected into 293T HEK cells with Flag-tagged SUMO1 or pcFlag. Cells were treated either T/I for 6 hrs or left unstimulated, as indicated in the figure. NEM was added in the lysis buffer. Flag antibody was used for immunoprecipitation followed by western blot detection by HA antibody. Arrow shows the SUMO-modified NFATc1 protein bands.

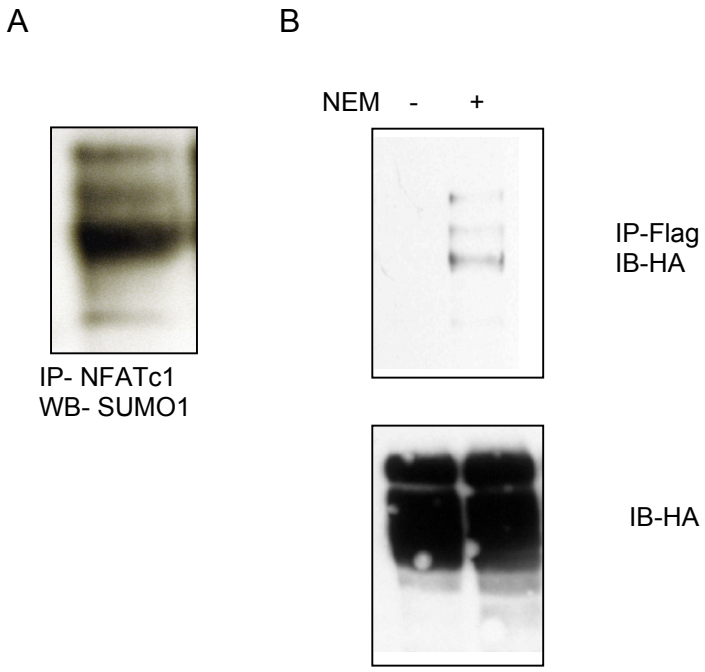



**Figure 3.6 Sumoylation of NFATc1 is highly transient**. **A**.Vector encoding HA-tagged c1/C was transfected into 293T HEK cells with Flag-tagged SUMO1. Cells were treated with T/I for 6 hrs. For immunoprecipitation a NFATc1 antibody was used followed by detection with SUMO1 antibody. **B.** Similar set of transfection as A, except lysis buffer was supplemented with or without NEM.

### *The Lysine 702 and 914 of NFATc1/C-specific C-terminus are potent sumoylation sites*

To analyze the individual sumoylation sites in c1/C, IP experiments were performed after co-transfecting 293T HEK cells with constructs encoding HA-tagged NFATc1/C, either wild type (wt) or different DSUMO-mutants of c1/C (ΔA - ΔE), and Flag-tagged SUMO1 expression vector. Wt c1/C produced three prominent and one uppermost weak SUMO-specific band (designated as 1-4 bands, Fig. 3.7). In case of ΔA (lane 2), bands 1 and 3 band persisted, whereas band 2 disappeared, suggesting the 2nd band as being specific for A-site sumoylation.

On the other hand, the 1st band was disappeared, indicating the band 1 as specific for B-site sumoylation. Furthermore, the overall sumoylation efficiency of ΔA and ΔB was drastically reduced compared to wt c1/C.

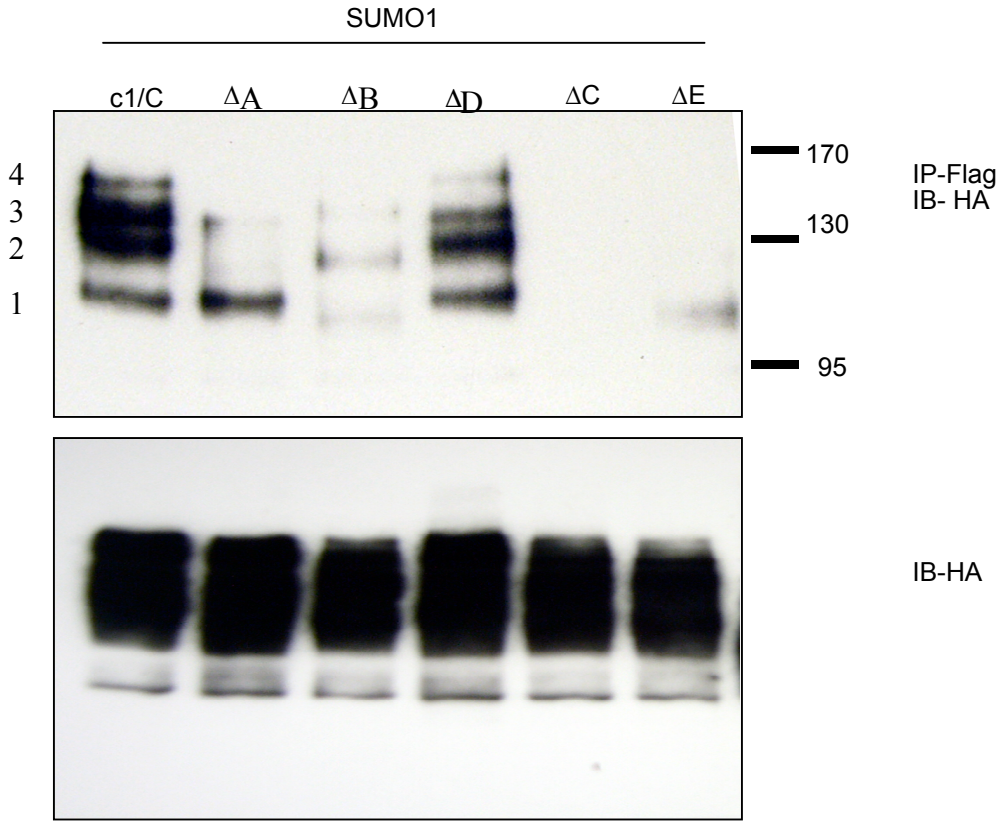



**Figure 3.7 Lysines 702 and 914 are the potent sumoylation sites on NFATc1.** Different DNA constructs encoding HA-tagged c1/C were transfected (as indicated into the figure) in 293T HEK cells with Flag-tagged SUMO1. After 48 hr of transfection The Cells were treated with T/I for 6 hrs. NEM was added to the lysis buffer. Flag antibody was used for immunoprecipitation followed by western blot detection by HA antibody.

Intriguingly, the SUMO-site mutant ΔD bearing a mutation in the "general" K 349 site gave rise to the same – although weaker - banding pattern as wt c1/C (lanes 1 + 4), implying that the strong SUMO-sites A and B at the C-terminus are the most prominent sumoylation sites, whereas sumoylation of D-site plays a minor role in c1/C sumoylation. To verify the dominance of A and B, both mutations were introduced in one construct (ΔC), and as expected, no sumoylated bands could be observed. This proved that the D-site is not important or may be totally dependent on C-terminal sumoylation (lane 5). According to the observation that without the C-terminal sites sumoylation is no longer possible, also no bands of sumoylated NFATc1/C could be detected in case of the triple DSUMO-mutant ΔE (lane 6).

We also examined sumoylation of the short isoform NFATc1/A which only harbours the "general" (D) SUMO-site. Suprisingly, we detected weak sumoylation of c1/A, which was abolished in the case of c1/ADD (Suppl-1). This indicates that the D-site is a true sumoylation site, although an extremely weak one. The latter is supported by the fact that no functional role could be pinpointed to this particular site (see below). Taken togaether, the long isoform-specific A- and B-sites are the most important sites for sumoylation, and mutation in any of those leads to dramatic reduction of overall sumoylation pattern.

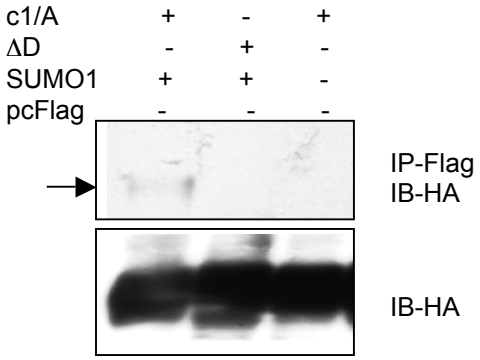



**Figure 3.8 The short isoform NFATc1/A shows very weak sumoylation**. Different plasmid constructs encoding protein (as indicated in the figure) were transfected in 293T HEK cells, either with Flag-SUMO1 or pcDNA. Cells were treated with T/I for 6 hrs. Immunoprecipitation by Flag antibody and western blot detection by HA antibody.

### *Sumoylation directs NFATc1/C to nuclear bodies*

It is a well-established fact that SUMO-modification of any protein alters its subcellular and/or subnuclear localization. SUMO-modified proteins colocalize with nuclear bodies, in human cells in particular with PML-nuclear bodies (PML-nbs). Therefore, we established stable cell lines from A3.01 cell, a human lymphoid T-cell line, by retroviral infection with viruses expressing c1/A, c1/C and c1/CΔE, respectively. In order to keep NFAT-proteins inactive, we fused the individual isoforms and mutants to part of the estrogen receptor (ER), which can be released by the treatment with 4-Hydroxy-Tamoxifen (Tm). Therefore, cells were activated with T/I plus Tm for 4 hrs and confocal microscopy experiments were performed. Before treatment, all NFATc1-ER proteins remained cytoplasmic (Fig. 3.9 a), while all translocated to the nucleus upon stimulation. Within the nucleus the short isoform c1/A, showing very weak sumoylation in IP, did neither colocalize with SUMO1 nor PML-nbs (Fig. 3.9, b). This is in clear contrast to the long isoform c1/C, which merges with SUMO1 as well as PML-nbs (Fig. 3.9, c), whereas the total SUMO-mutant ΔE does not colocalize with SUMO1 nor PML-nbs (Fig. 3.9, d). Therefore, strong sumoylation directs c1/C into PML-nbs. Supporting the established notion of matured PML-nbs, SUMO1 always colocalized with PML-nbs, which was independent of NFATc1. Hence, upon sumoylation NFATc1/C is directed to mature PML-nbs.

To provide further evidence that sumoylation is solely responsible for colocalizing with PML-nbs, SUMO-fused NFATc1 (S-NFATc1) encoding constructs were generated and transfected into 293T HEK cells. Now, all different NFATc1-proteins (S-c1/A, S-c1/C and S-c1/CΔE), irrespective of the short, long and SUMO-site mutant, merged with SUMO1 as well as PML-nbs, indicating that SUMO-modification is a hallmark for sequestration of NFATc1 into PML-nbs (Fig. 3.10).

### *Sumoylation represses NFATc1 transcriptional activity*

We next sought to examine whether sumoylation exerts any impact on NFATc1 function. In order to pursue this objective, a luciferase-reporter gene activity assay was performed using the IL-2 promoter as transcriptional target of NFATc1.

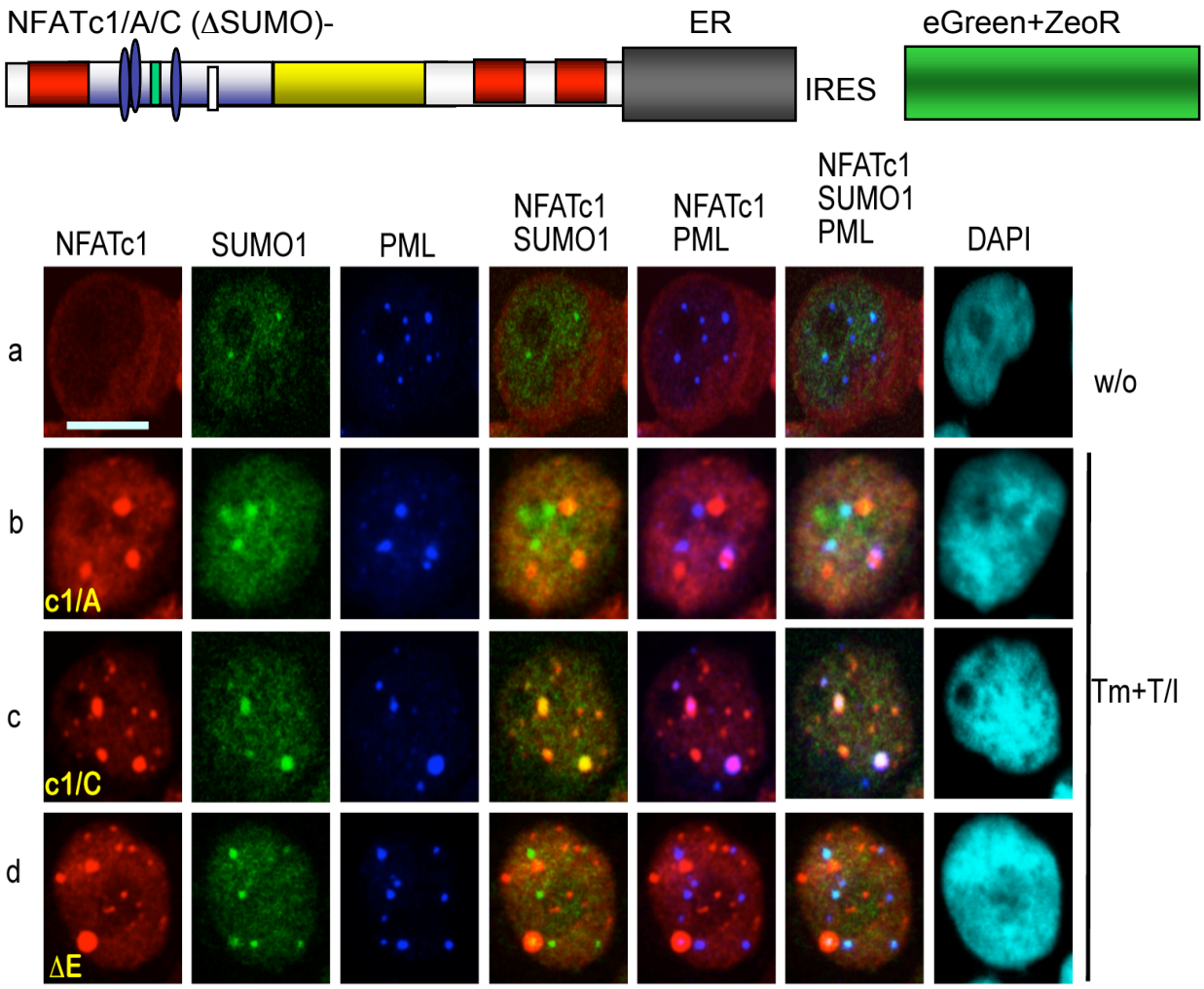



**Figure 3.9 Sumoylation directs NFATc1 into nuclear bodies.** A3.01 cells were infected with retroviruses expressing c1/A, c1/C and ΔE mutant. HA-ER expressing virus was also introduced in the infection experiment as an empty vector control. The infected cells, after selection, were stimulated with Tm+T/I for 4 hrs. After stimulation, immunofluorescence staining was performed with ER (to detect exogenous NFATc1), SUMO1 and PML antibodies followed by laser scanning confocal microscopic analysis. The scale bar represents 10 μm in length.

A strong upregulation of reporter gene activity compared to wt NFATc1/C was observed by any of the C-terminal SUMO-site mutants (Fig. 3.11, A). Accordingly, in immunofluorescence experiments, a dramatic (around 40%) reduction of colocalization between ΔA or ΔB with SUMO1 was detected (data not shown), indicating SUMO1-containing bodies as transcriptional repressor sites in the context of NFATc1 sumoylation

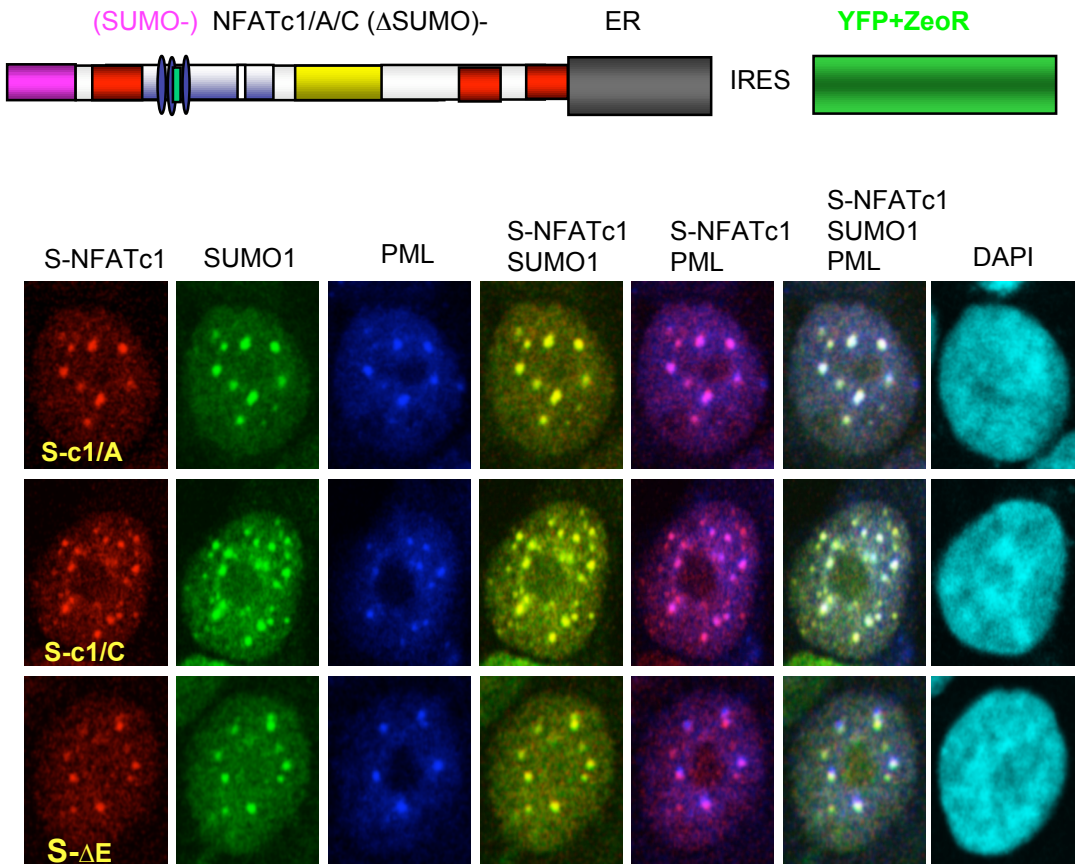



**Figure 3.10 Sumoylation of NFATc1 is indispensable for co-localization with PML-nbs.** 293T cells were transfected with c1/A, c1/C and ΔE. All the NFATc1 proteins were fused with SUMO1 at the N-terminus and YFP at the C-terminus. Cells were treated with T/I/$CaCl_2$ for 1 hr, followed by immunofluorescence staining (SUMO1 and PML antibody) and analysis by laser scanning confocal microscopy.

Reporter gene activity of ΔD was always similar to that of wt c1/C, which is in line with the fact that the extent of ΔD-sumoylation is similar to wt c1/C. If both C-terminal or all three SUMO-sites were mutated, as in ΔC or ΔE, the transactivation potential increased even further. It is important to note that no significant difference between c1/CΔC and c1/CΔE mutant in localization and function was observed, confirming that the D-site is dispensable for NFATc1 function. Moreover, fusing SUMO with transcriptionally hyperactive ΔE, resulting in SUMO-DE, led to a dramatic downregulation of its transactivation potential (Fig. 3.11, B), identifying this functional effect on

c1/C as sumoylation-mediated. Furthermore, no difference in transactivation potential of c1/A and c1/AΔD mutant was detected, confirming that there is no functional effect of weak sumoylation on the short isoform c1/A (data not shown). When T cell lines were transfected with the same constructs – for example the murine thymoma cell line EL4 - similar results were obtained: when SUMO1 was overexpressed it could exert a negative effect on wt NFATc1/C, but not on the ΔE-mutant which again was very active in transactivation (Fig. 3.11, C). Taken together, sumoylation of the C-terminus, specific for the long isoform, exerts a negative effect on NFATc1 function.

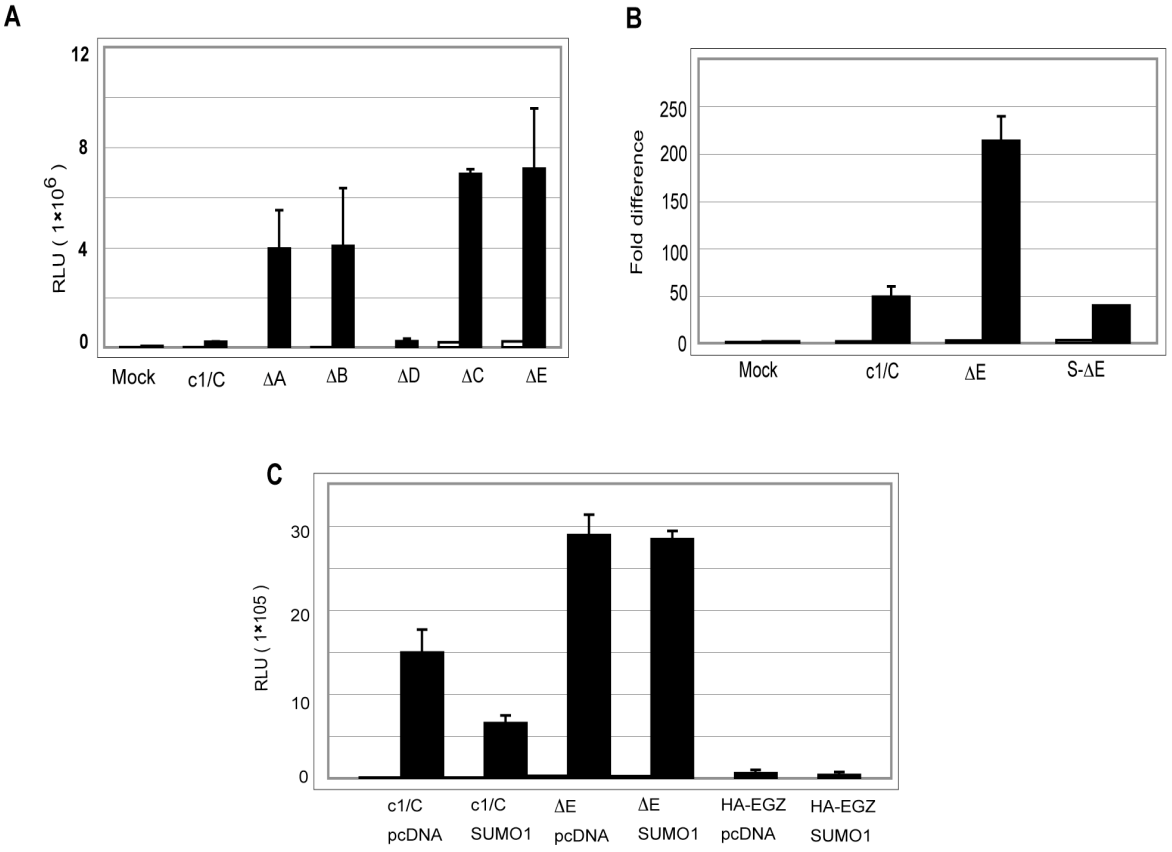



**Figure 3.11 Sumoylation represses NFATc1 transcriptional activity.** ***A.*** 293T HEK cells were transfected (by the calcium-phosphate method) with 10 μg of plasmid DNA (EGZ-HA-NFATc1/C or SUMO site mutants of NFATc1 along with a luciferase reporter plasmid (under control of *IL-2* promoter). After 36 hr of transfection luciferase activity was measured from the cells that were either left untreated or treated with TPA/ionomycin for overnight. Normalized mean values of three independent experiments were taken. Error bars of SEM are shown. Western blot was performed from the protein extracts to check the equal expression pattern of transfection. ***B.*** Similar as A, except that SUMO1-fused NFATc1 clones were

used for the transfection. ***C.*** Similar as A, except EL4 cell line was used for the assays and the DEAE dextran method was followed for the transfection.

### *Sumoylation makes NFATc1/C less potent on endogenous IL-2 expression*

To test the physiological effect of sumoylation on NFATc1 function on endogenously expressed IL-2, the retrovirally transduced A3.01 cells were assayed in RNase protection assay (RPA) (Fig. 3.12).

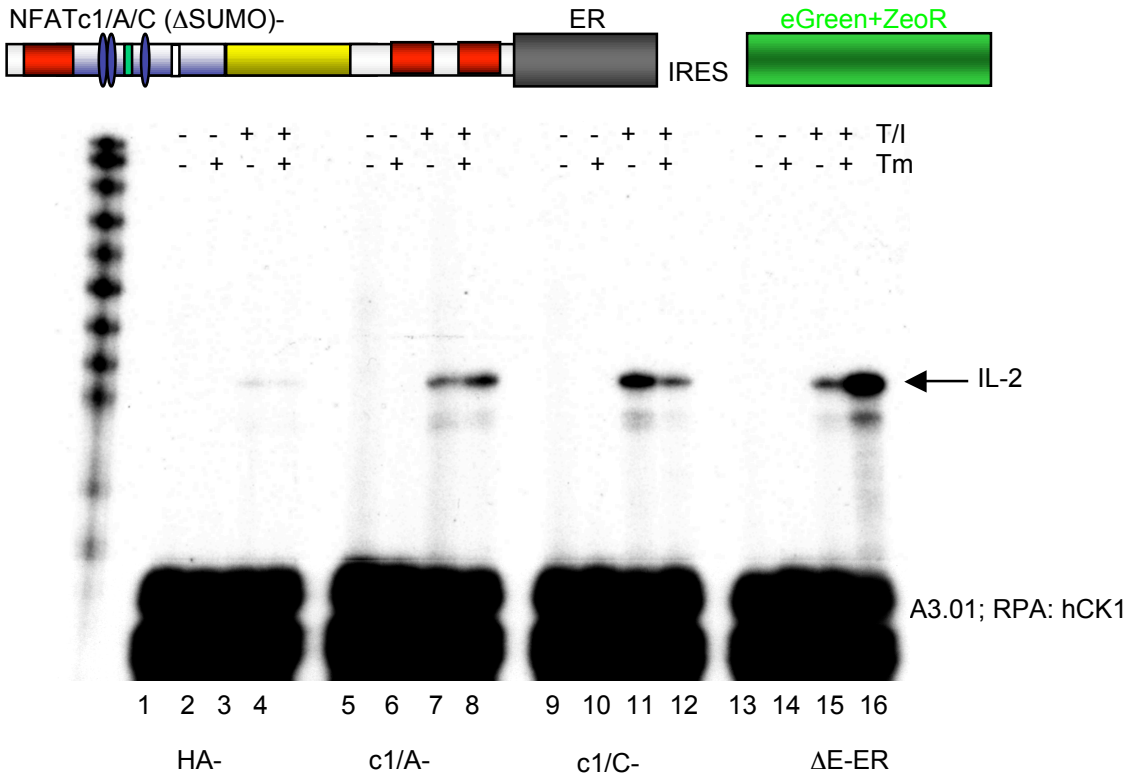



**Figure 3.12 Suppression of endogenous IL-2 mRNA expression upon NFATc1 Sumoylation.** Retroviral infected A3.01 cells were stimulated by Tm+T/I for overnight. RNA was isolated. An RNase protection assay (RPA) was performed to study the effect of NFATc1 sumoylation on the synthesis of endogenous IL-2 mRNA transcripts.

Cells were again stimulated by T/I+Tm, which gives full activation (lanes 4, 8, 12, 16). A less positive effect of wt NFATc1/C compared to c1/A on endogenous IL-2 mRNA

level could be demonstrated, but strikingly, a further upregulation of endogenous IL-2 level was found in the presence of c1/CΔE which was even far stronger than the transactivation by the short isoform. This observation falls in line with the reporter assays. When in RPA assays, cells were also left untreated (lanes 1, 5, 9, 13) or solely given Tm (lanes 2, 6, 10, 14), this gave no raise to any transactivation by NFATc1 proteins. To our surprise, when cells were stimulated by T/I only (lanes 3, 7, 11, 15) some obscure NFATc1 activity was observed. Thus, we checked the nuclear translocation of NFATc1 in confocal microscopy after different combination of stimuli. Although nuclear translocation of NFATc1 could be achieved by T/I only, the T/I stimulus did not provide the typical punctuated distribution of NFATc1 within the nucleus (Fig. 3.13). This kind of distribution was achieved only when cells were treated with both T/I and Tm, suggesting that the subnuclear distribution of NFATc1 is only possible when the fused ER is released by estrogen. This demonstrates the importance of naturally occurring, SUMO-mediated subnuclear localization for fine-tuning of NFATc1's activity.

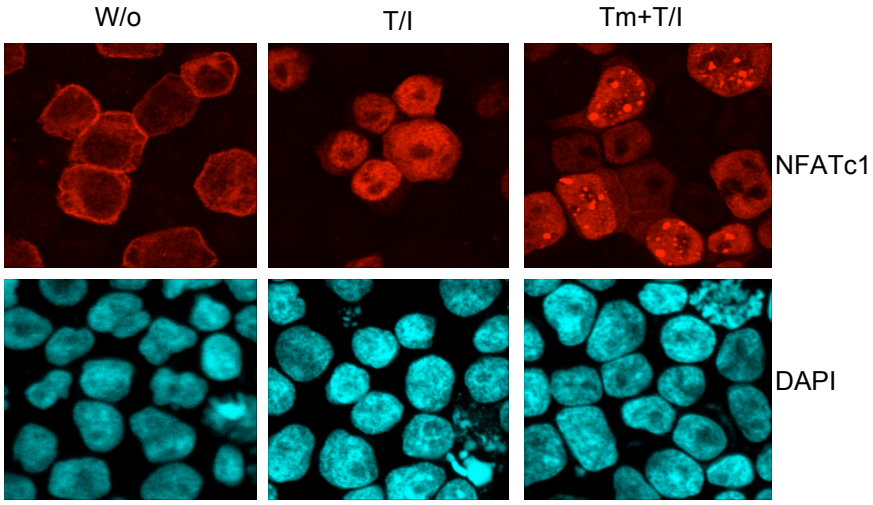



**Figure 3.13 T/I is sufficient for NFATc1 nuclear translocation, but not for proper subnuclear localization as and activity.** To check the effect of T/I and Tm, nuclear translocation studies were performed by using retroviral infected A3.01 cell line. The cells were treated with T/I, Tm+T/I for 4 hrs or left untreated. NFATc1 was stained by an ERα antibody and the nuclear translocation of NFATc1 was analyzed by confocal microscopy.

Apart from mRNA level, a dramatic reduction (4x) in IL-2 protein level in the presence of the sumoylatable long isoform and a 8-fold increase upon the eradication of SUMO-sites (DE) could be demonstrated by ELISA (Fig. 3.14). Taken together, we have established that upon sumoylation, NFATc1/C redistributes to PML-nbs and is less active as transcriptional transducer on IL-2 gene expression.

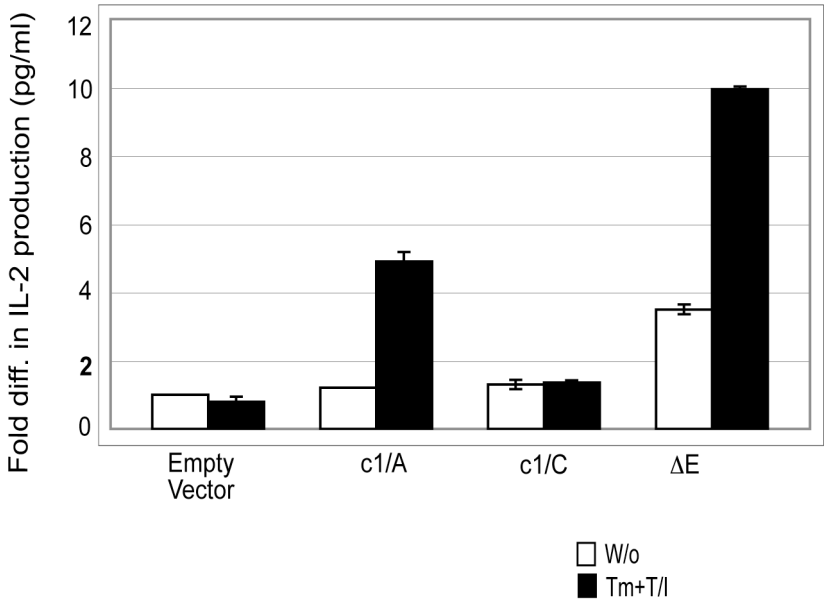



**Figure 3.14 Sumoylation of NFATc1 leads to dramatic reduction of endogenous IL-2 production** A3.01 cells were infected with retroviruses expressing c1/A, c1/C and ΔE mutant. HA-ER expressing virus was also introduced in the infection experiment as an empty vector control. The infected cells, after selection, were stimulated with Tm+T/I for overnight. Endogenous IL-2 production was measured by ELISA.

### *SUMO1 colocalizes with NFATc1 in primary CD4$^+$ T cells*

Next, sumoylation of NFATc1 was checked in primary CD4$^+$ T cells. Since NFATc1/C sumoylation leads to suppression of IL-2 gene activity and thereby IL-2 production, naturally occurring regulatory T cells (nTregs, CD4$^+$ 25$^+$), which do not produce IL-2, were an interesting candidate to include. Thus, we performed immunofluorescence experiments on *in vitro* restimulated conventional CD4$^+$ effector T cells and CD4$^+$ 25$^+$ nTreg cells by using an anti-

NFATc1 antibody, which recognizes the N-terminal part of NFATc1, i.e. all NFATc1 isoforms. Both $CD4^+$T cells expressed NFATc1 as well as SUMO1, and in both endogenous NFATc1 colocalizes with SUMO1 bodies (Fig. 3.15, upper panel). Interestingly, NFATc1 signals in nTregs were always reduced (Fig. 3.15, lower panel). More excitingly, the extent of SUMO1-colocalized NFATc1 was more pronounced. Thus, the maintenance of anergic phenotype of nTreg cells might be supported by the higher sumoylation level of c1/C, which, thereby, suppresses IL-2 production.

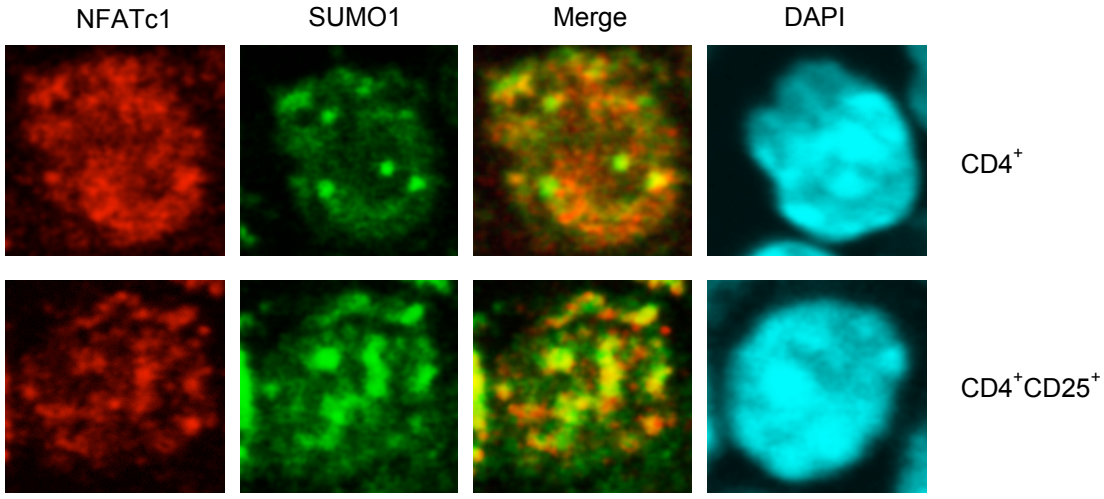



**Figure 3.15 Endogenous NFATc1 colocalization with SUMO1 in primary mouse T cells.**

$CD4^+$ and $CD4^+CD25^+$ T cells were isolated from mouse (black 6 strain) lymph nodes. Cells were stimulated with plate bound (2-5 million cells/ well) anti-CD3 and anti-CD28 antibody for 3 days. After that, cells were kept for resting stage (without any antibody) for 4 days. Cells were then restimulated with T/I for 6 hrs before cytospin. Immunofluorescence staining was performed with NFATc1 and SUMO1 antibody followed by confocal microscopy analysis.

### *Sumoylation of NFATc1 exerts differential regulation on endogenous lymphokine genes*

Since many of the other lymphokine genes are also under direct control of NFAT, we further asked if this negative functional effect of sumoylation is a general phenomenon. Therefore, murine EL-4 cells, which express Th1 as well as Th2 lymphokine genes upon stimulation, were retrovirally infected virus expressing with NFATc1-ER proteins. After treatment with T/I+Tm for 24 hrs, RNA was isolated for RPA. Again, ΔE exerts a much stronger transactivation potential on IL-2 mRNA as wt NFATc1/C (Fig. 3.16, lanes 2+4). The same is true for ΔC, confirming the C-terminal SUMO-sites as necessary and sufficient (lane 3). Intriguingly, all other lymphokines tested, i. e. Th1 (IFN-γ) and Th2 (IL-4, -5, -10 and -13) exhibited reduced transcript levels under the control of non-sumoylated NFATc1/C. Accordingly, when SUMO was fused to wt NFATc1/C (S-c1/C), especially the amount of IFN-γ and IL-13 transcript was robustly increased above wt-mediated levels (Fig. 3.16, lanes 2+5). This suggests a dependence of NFATc1 being sumoylated for full-fledged IFN-γ and IL-13 gene expression and the opposite behaviour as for IL-2. Since all SUMO-fused NFATc1 proteins, irrespective of intact endogenous SUMO-sites, localized to PML-nbs, S-c1/C and S-ΔE were expected to have a similar impact on cytokine production profile. Surprisingly, although IL-2 mRNA level was reduced in case of S-ΔE compared to ΔE (Fig. 3.15, lanes 4+6), it was still enhanced in comparison to SUMO-fused wt (lanes 5+6). Furthermore in case of IFN-γ and IL-13 RNA-expression, the loss of the SUMO-sites within the NFAT-protein dramatically dominated over the presence of the N-terminally fused SUMO (lanes 5+6). This result indicates that the position of sumoylation on NFATc1 is one of the determining factors for its functional outcome.

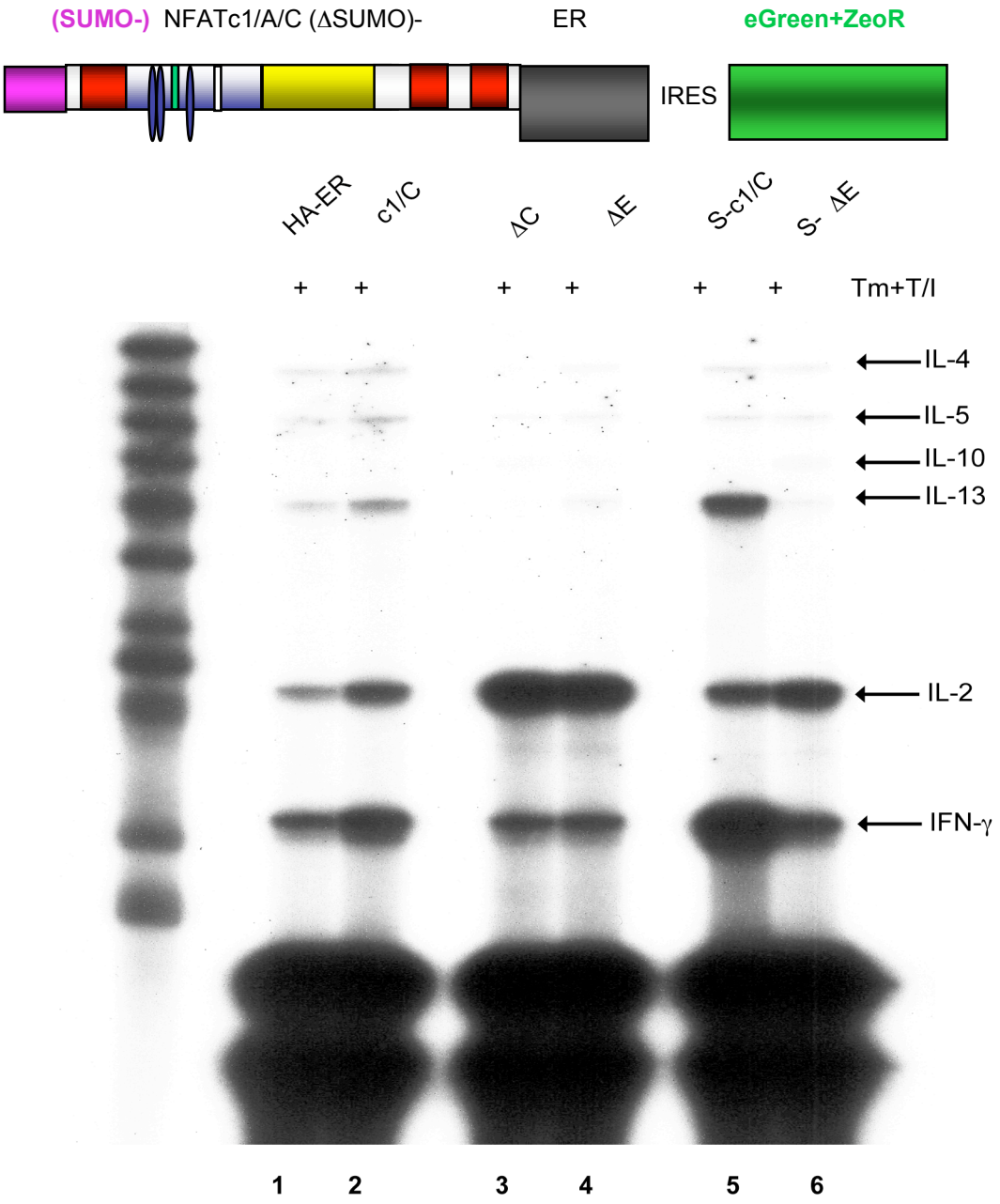



**Figure 3.16 Differential regulation of cytokine gene expression by NFATc1 sumoylation.** EL4 cells were infected with retroviruses encoding different forms of c1/C. A brief description of NFATc1 vector map is given above. Cells were treated with Tm+T/I overnight. RNA was harvested and analyzed in a RNase protection assay (RPA).

### *SUMO1 promotes NFATc1 interaction with HDACs and reduces histone acetylation level on IL-2 promoter*

Earlier studies described that SUMO1-modified transcription factors can associate with the transcriptional repressor HDAC2 (Yang et al., 2004). Therefore, we argued that the repression of NFATc1/C's transactivation potential upon sumoylation could be mediated through HDAC2. IP experiments were performed after co-transfecting 293T HEK cells with Myc-tagged HDAC2 and HA-tagged NFATc1 encoding plasmids. And indeed, HDAC2 interacts with c1/C (Fig. 3.17, A, lane 2). This interaction was drastically reduced or abolished in case of c1/A and ΔE, suggesting that SUMO1-modification on NFATc1 serves as a tag for interaction with HDAC2 (lanes 1+3). Furthermore, we established that c1/C, but not DE, can also be efficiently precipitated by HDAC1 and HDAC4, meaning by both class-I as well as class-II HDACs (Fig. 3.17, B). As sumoylation promotes c1/C and HDACs interaction, this event could lead to deacetylation of histones at the IL-2 promoter making chromatin less accessible for transcription. To test this, Chromatin Immuno Precipitation (ChIP) assays were performed with a "general" anti-acetylated histone 3 antibody (mark for active chromatin in) making use of those retrovirally transduced A3.01 cells. To amplify the immunoprecipitated chromatin, an oligonucleotide primer pair specific for the human IL-2 promoter was used in PCR. In case of c1/C-expressing cells a dramatic reduction in the level of histone acetylation on the IL-2 promoter could be demonstrated, while it was comparably high in both c1/A as well as ΔE (Fig. 3.18, left panel). When subsequently an anti-ER antibody was applied to precipitate exogenous NFATs, equal binding of c1/C and ΔE to the IL-2 promoter could be detected (Fig. 3.18, right panel). This is in line with electromobility shift assays (EMSA), which elucidated the same binding capacity of NFATc1 *in vitro* irrespective of isoform and state of sumoylation (data not shown). Taken together, these data proved that HDACs are recruited to the IL-2 promoter by sumoylated NFATc1/C, thereby clearing marks of active chromatin.

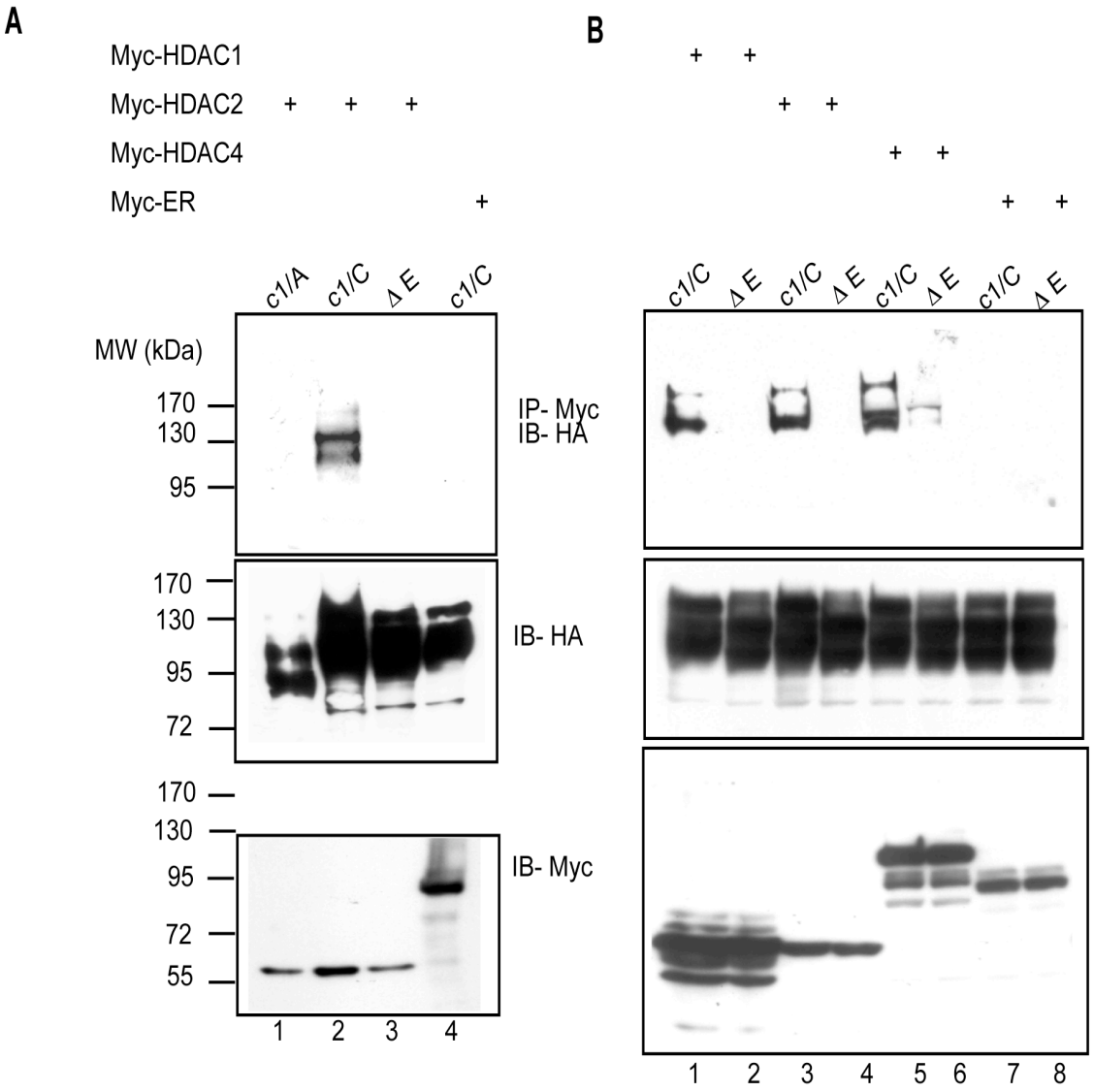



**Fig. 3.17 Sumoylation promotes NFATc1 association with transcriptional repressors of HDAC family (class I and II).** ***A.*** 293T HEK cells were transfected with Myc-tagged HDAC 2 construct and different forms of NFATc1, which are HA-tagged. After 48 hrs of transfection, cells were treated with T/I for 6 hr. Cells lysis was done in presence of NEM in the lysis buffer. Myc-antibody was used for immunoprecipitation, followed by HA antibody for western blot detection. ***B.*** Same as left panel, except HDAC1 and HADC4 constructs were also used in transfection (in addition to HDAC2) followingly IP.

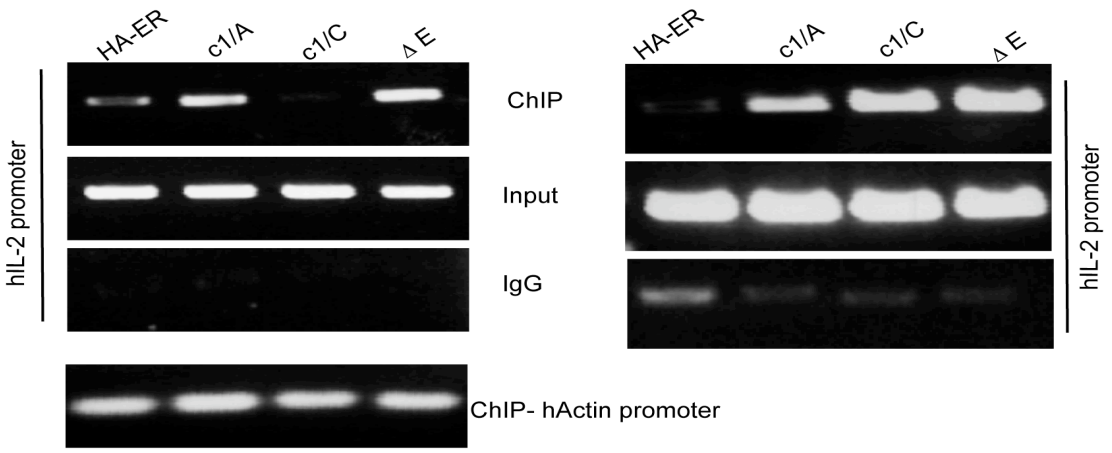



**Figure 3.18 Recruitment of HDACs upon NFATc1 sumoylation leads to deacetlylation of IL-2 promoter chromatin.** *Left panel,* retroviral infected A3.01 cells (HA-ER, c1/A, c1/C and ΔE, as described in Fig 3.12) were stimulated with Tm+T/I for 6 hrs. Chromatin was isolated and an equal amount of chromatin was taken. Anti-H3-Ac ("general" acetylated H3 antibody) antibody was used for chromatin immunoprecipitation (ChIP). DNA was extracted from the immunorecipitated chromatin and amplified in PCR using oligonucleotide primers for the amplification of the hIL-2 promoter; hActin promoter was also used as an internal control. *Right panel,* same as left panel except, an anti-ERα antibody was used for ChIP.

### *SUMOylation withdraws NFATc1 from transcriptional hotspots and localizes it to condensed chromatin*

Recruitment of HDACs by SUMO-modified NFATc1 results in lower acetylation level on the IL-2 promoter. In other words, this event might leave a signature for close chromatin. One of the best characterized mark for closed chromatin is histone 3 lysine 9 (H3K9) trimethylation (m3). It has also been shown that sumoylation directs its target protein to the pericentric heterochromatin which are enriched by H3K9 m3 marks and redistribution of any protein to those chromatin domain has been correlated with its function as a repressor or silencer of gene expression (Berberich-Siebelt et al., 2006, Avitahl et al., 1999, Kim et al., 1998). Therefore, we examined if upon sumoylation c1/C co-localizes with H3K9 m3 marks. In three independent immunofluorescence experiments each time roughly 100 cells were analyzed in detail, elucidating 56% of c1/C, but only 29% of c1/A and 31% of ΔE within condensed chromatin (Fig. 3.19). Confirming the role of SUMO as the determining factor for directing NFATc1 to repressed chromatin, all SUMO-fusion constructs (S-c1/A, S-c1/C and S-ΔE) were detected exclusively at H3K9 me-positive sites (Fig. 3.20, A).

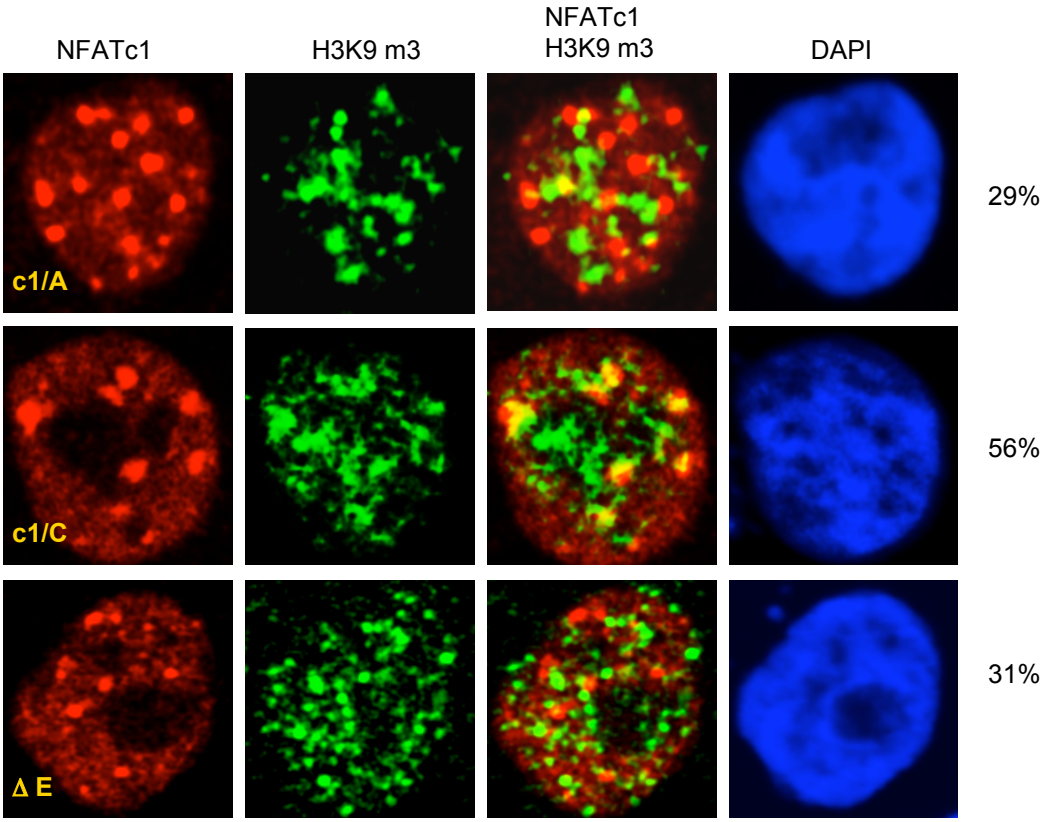



**Figure 3.19 Sumoylation directs NFATc1 into heterochromatin.** 293T HEK cells were transfected with c1/A, c1/C and ΔE plasmid DNA. After 48 hrs of transfection, cells were stimulated with T/I/$CaCl_2$ for 1hr. Immunofluorescence staining was performed with mouse HA (to detect NFATc1) and rabbit H3K9m3 (to detect heterochromatin) antibody. Colocalization of NFATc1 and heterochromatin was analyzed by laser scanning confocal microscopy.

Moreover, to question the presence of SUMO1 at condensed chromatin, NFATc1 colocalization studies with anti-H3K9 m3 as well as anti-SUMO antibodies were performed. A similar outcome, demonstrating the wild type long isoform to colocalize with SUMO1 and H3K9 m3 in a substantial amount, verified SUMO1 as a tag for subnuclear orientation to sites of repression (Fig. 3.20, B).

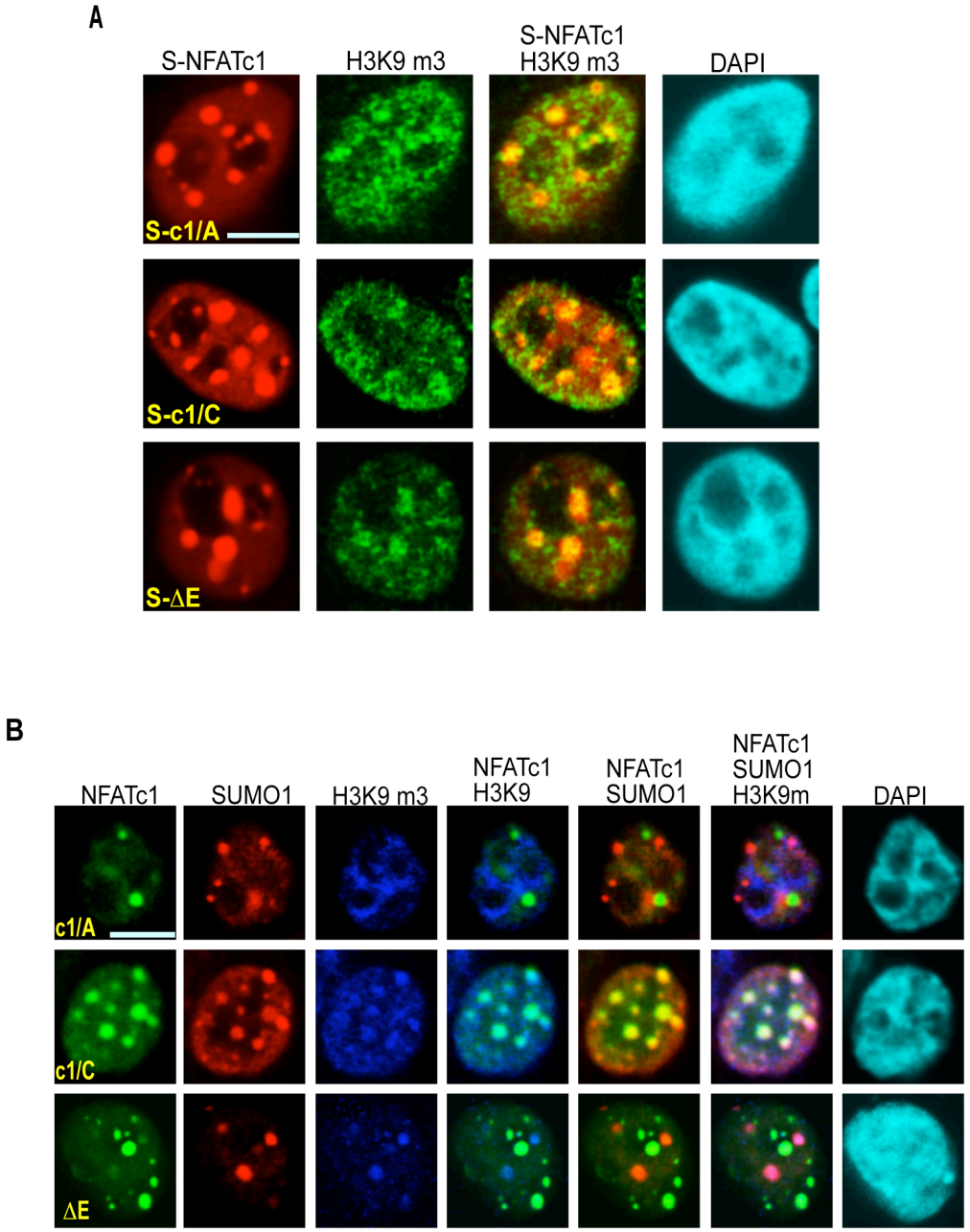



**Figure 3.20 SUMO serves as molecular tag for NFATc1 co-localization with heterochromatin.**

***A.*** 293T HEK cells were transfected with c1/A, c1/C and ΔE plasmid DNA. All the NFATc1 proteins have been fused with SUMO1 at the N-terminus and YFP fusion at the C-ternimus end. After 48 hrs of transfection, cells were stimulated with T/I/$CaCl_2$ for 1hr. Immunofluorescence staining was performed with rabbit H3K9m3 (to detect heterochromatin) antibody.

Colocalization of S-NFATc1 and heterochromatin was analyzed by laser scanning confocal microscopy. ***B,*** c1/A, c1/C and ΔE plasmids were transfected into 293T HEK cells along with Flag-SUMO1. NFATc1 proteins contain YFP as a fusion peptide. After 48 hrs of transfection, cells were stimulated with T/I/$CaCl_2$ for 1hr followed by immunofluorescence staining with mouse SUMO1 (to detect SUMO1 bodies) and rabbit H3K9m3 (to detect heterochromatin) antibody. Confocal microscopic analysis was performed to check NFATc1, SUMO1 and H3K9m3 tri-colocalization.

Strikingly, also NFATc1 in its active forms (c1/A and ΔE) established a punctated distribution within the nucleus. Previously, it has been shown that numerous transcriptional hotspots exist within the nucleus and that those spots contain various kinds of transcriptional modulators. In this context RNA pol II is the best characterized protein and forms so-called RNA pol II bodies (Osborne. 2004).

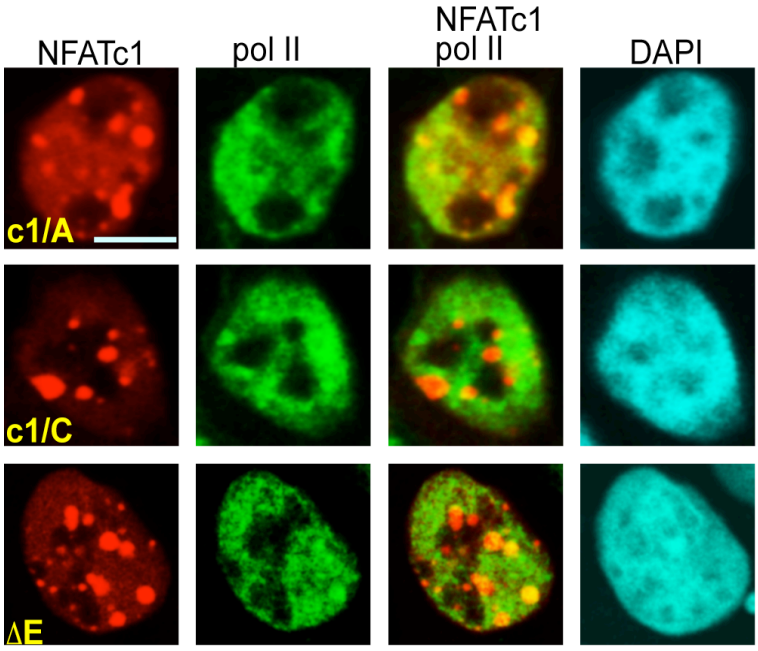



**Figure 3.21 The short isofom NFATc1/A and SUMO-site mutant ΔE, but not c1/C, partially colocalizes with RNA pol II bodies.** 293T HEK cells were transfected with c1/A-, c1/C-, ΔE- YFP fusion constructs. After 48 hrs of transfection, cells were stimulated with T/I/$CaCl_2$ for 1hr. Immunofluorescence staining was performed with mouse monoclonal RNA pol II antibody to detect pol II bodies. Laser scanning confocal microscopic analysis was performed to study the co-localization pattern of NFATc1 and RNA pol II bodies.

Intriguingly, by colocalization experiments where NFATc1 was co-stained with RNA pol II antibodies, we found that c1/A and ΔE, but not c1/C, partially colocalizes with pol II bodies (Fig. 7D). This indicates that the transcription factor NFATc1 associates with the highly active transcription machinery and that sumoylation can withdraw NFATc1 from those "transcriptional factories".

# 4. DISCUSSION

Our study underlines a novel regulatory mechanism by which NFATc1 function is modulated. Apart from phosphorylation, sumoylation adds an additional posttranslational modification of NFATc1, which has important functional consequences. First of all, sumoylation alters the subnuclear localization of NFATc1 molecules. Secondly, the attachment of SUMO moiety to NFATc1 creates an additional surface for protein-protein interactions such as HDACs, and modulates chromatin organization to some of its target genes like IL2, leading to transcriptional repression.

In the peripheral blood, Naïve $CD4^+$ T cells can differentiate into several distinct subsets such as, in Th0, Th1, Th2, Th17 and subsets of induced regulatory T cells (Weaver et. al. 2006). The various subpopulations are defined by functional abilities and different cytokines produced. Like all other $CD4^+$ T cells, $CD4^+CD25^+$ regulatory T cells, thymus-derived as well as induced, are highly dependent on, but do not produce IL-2 themselves. In general, lymphokine expression in T cells including IL-2 is controlled to a substantial degree by the action of NFAT transcription factors (Serfling et al., 2000).

NFAT ("nuclear factor of activated T cells") proteins belong to a family of transcription factors whose genuine members are designated NFATc1 – 4. NFATc1 and NFATc2 are highly expressed in peripheral T cells and control their effector function. Besides lymphokine promoters further targets which are bound and controlled the promoters of $p21^{Waf1}$, *CD40* ligand, *CD95* ligand and *NFATc1* itself. Different from transfection studies, though it could be demonstrated by knock-out mice that NFATc1 and c2 have divergent functions in lymphokine gene expression (Serfling et al., 2000). While $NFATc2^{-/-}$ mice suffer from lymphoproliferative disorder and a strong – though may be indirect - induction of Th (T helper) 2 lymphokines, peripheral T-cells from $NFATc1^{-/-}$ mice are defective in proliferation and Th2 lymphokine expression. This indicates contrasting roles on T-cell proliferation and activation with NFATc2 being negative for T-cell activation for expample in IL-4 expression, but NFATc1 being indeed positive for both. The effects on IL-2, the lymphokine important for proliferation, are negligible in case of $NFATc2^{-/-}$ and confusing for $NFATc1^{-/-}$, i.e. one of two strains synthesized more IL-2 after

secondary stimulation (Ranger et al., 1998) raising the question if under some circumstances NFATc1 could have a negative effect on IL-2 expression.

NFATs share a common DNA binding domain (Serfling et al., 2000). This domain is very similar in its conformation to the Rel DNA-binding domain of Rel/NF-κB factors and, therefore, was designated as Rel similarity domain (RSD). The N-terminus of NFATc proteins harbor a TAD (transactivation domain), and a regulatory domain. The latter is decisive for nuclear import and export of the genuine NFAT factors (NFATc1 – 4) which is regulated by phosphatases and kinases, especially by the $Ca^{2+}$/calmodulin-dependent phosphatase calcineurin (Crabtree, 1999). NFATs are expressed in multiple isoforms with variation of the very N-terminal as well as considerably in the C-terminal ends. The short isoform of NFATc1, designated as NFATc1/A, is highly induced upon activation of T-cells (Chuvpilo et al., 2002; Chuvpilo et al., 1999b). Interestingly, the longest isoform of NFATc1, called the C-isoform and expressed constitutively, harbors an extra C-terminal peptide with a second TAD, TAD-B, which is very homologous to the C-terminus of NFATc2 (Chuvpilo et al., 1999a). Therefore, the question rises if NFATc1/C functionally resembles NFATc2 or has a function of its own, i.e. whether NFATc1/C is not only different from NFATc1/A, but also from NFATc2.

In a yeast two-hybrid screen, the regulatory proteins (Ubc9, PIAS1 etc.) of sumoylation pathway were identified as partners of long isoform of NFATc1. Sumoylation, in general, brings a negative impact on its target protein activity. Therefore, we argued that the long isoform might have a negative impact on cytokine production, due to its modification by SUMO.

Functions of proteins can be affected by post-translational modifications, for example by SUMO (Small Ubiquitin-related Modifier). Similar to ubiquitinylation, sumoylation is mediated by activating E1, conjugating E2 and ligating E3 enzymes. Sumoylation occurs at the consensus motif Ψ-Lys-x-Glu (where Ψ is a large hydrophobic aa, generally isoleucine or valine, and x any residue) which is most likely in the neighborhood of a stretch of prolines (Gill, 2004; Iniguez-Lluhi and Pearce, 2000). So far, four SUMO family members, SUMO-1/Smt3C, SUMO-2/Smt3A, SUMO-3/Smt3B, and SUMO-4 are known to exist in mammals. Sumoylation often correlates with relocalization of the targeted proteins, which means towards PML (Promyelocytic leukemia) nuclear bodies (Dobreva et al., 2003; Ross et al., 2002; Sachdev et al., 2001; Schmidt

and Muller, 2002) or heterochromatin (Berberich-Siebelt et al., 2006). Accordingly, although sumoylation of transcription factors might enhance activity, mostly it represses the inactivation potential (Gill, 2005).

**Ubc9 is sufficient to induce NFATc1 sumoylation, but depends on intact SUMO-sites.**

The long isoform of NFATc1 (c1/C) can interact physically with Ubc9, which we precipitated in yeast two hybrid screen, as a NFATc1-binding partner, confirming a true positive interaction between both partners. However, no interaction between SUMO1 and c1/C was observed in the yeast two hybrid screen. This could be due to the presence of low SUMO conjugates in yeast cells at a given time. From the *in vivo* interaction study (IP), between Ubc9 and c1/C, we found the appearance of more than one band, although Ubc9 interacts non-covalently with its partner. This observation suggests that the Ubc9 alone is sufficient to catalyze sumoylation of NFATc1 and the extra upper bands are the mono-sumoylated NFATc1 bands. Our argument was confirmed by introducing the ΔE mutant in an IP experiment. In this set up, we did not observe any SUMO-modified NFATc1 bands. Thus, intact SUMO-1 consensus motifs of NFATc1 are necessary for the proper interaction with Ubc9. This finding is consistent with the previous study demonstrating that the SUMO-1 consensus sequence mediates Ubc9 binding which is essential for SUMO-1 modification (Sampson. 2001). Additionally, it is also clear that Ubc9 is necessary but not enough to induce efficient NFATc1 sumoylaion.

**Sumoylation of NFATc1 is a highly dynamic and transient process**

In the *in vivo* experimental set up (IP), we have always detected 3-4 sumoylated NFATc1 bands. Depending on the number of sumo sites on NFATc1, theoretically one could expect 7 bands. This is due to mono-sumoylation of three sites, di- and tri-sumoylation. But, practically this was not consistent with our observations. In most of the experiments, we detected the mono sumoylation bands (3 bands) and in a few instances an additional SUMO-modified c1/C band was precipitated. This finding suggests that, in order to achieve di- or tri-sumoylation, all the parameters of the sumoylation machinery have to be in a perfect balance. A robust c1/C sumoylation was always observed when the lysis buffer was supplemented with NEM (a SUMO

protease inhibitor), which indicates the highly transient nature of sumoylation. These data also suggest that a perfect molecular balance between the SUMO conjugating and deconjugating activities are necessary for c1/C sumoylation. This interplay might be mediated through a fine-tuning of some specific signalling.

NFAT is usually heavily phosphorylated and stays in the cytosol (inactive state). Upon T/I stimulation, it gets dephosphorylated and translocates to the nucleus. The dephosphorylated state of NFAT is the active state. We exploited the phospho-dephosphorylated state of c1/C and wanted to check if phosphorylation has any impact on c1/C sumoylation. This argument was in light of the fact that, the sumoylation can be favoured or antagonized by other modification such as phosphorylation, ubiquitinylation etc (Gre´goire et. al., 2005, Desterro et.al. 1998). Therefore, cells were treated with T/I or left unstimulated. In IP, we detected c1/C sumoylation under both stimulated as well as unstimulated condition. But, the sumoylated c1/C bands were found to be enriched upon T/I stimulation. Thus, we conclude that dephosphorylation is needed for efficient c1/C sumoylation. Sumoylation of c1/C in its phosphorylated state may not give any important functional output, since the phosphorylated form of NFAT is not known to play any functional role. This explanation falls in the line of our reporter gene assays and RPA, in which the differential transactivation potential of c1/C and SUMO-site-mutant c1/C were observed only after T/I treatment.

**The C-terminal SUMO sites of c1/C determines its sumoylation**

*In silico* analyses predicted that lysine 702 (A-site) and 914 (B-site) are the two strong sumoylation sites residing in the C-terminal domain of c1/C. On the contrary, the "general" SUMO site around ysine 349 (D-site) was predicted as a weaker sumoylation site. This prediction was confirmed in the IP, in which we found that the deletion of lysines 702 and 914 (ΔC-mutant) abrogated the sumoylation of c1/C. It suggests that the D site sumoylation is dependent exclusively on the sumoylation of A and B sites, at least in the context of the long isoform (c1/C). This could be due to the tight and stable docking of sumoylation machinery on its target proteins only in the presence of strong SUMO consensus sites. Accordingly, mutation of either the C-terminal SUMO site on the C-terminus resulted in a dramatic reduction of overall

sumoylation, implying both lysines of the C-terminus are required for robust sumoylation of c1/C. The ΔE mutant (all SUMO-site mutant of c1/C) behaved like ΔC, not any band in IP indicating those as the actual sumo sites on c1/C. However, in some IP experiments, we observed a weak precipitation in ΔE mutant lane. Therefore, some cryptic SUMO sites might exist on c1/C, which we might have overlooked. However, since the ΔE mutant was sufficient to relieve the negative functional effect of sumoylation on c1/C, those cryptic SUMO sites might not posses any functional impact. As we have concluded that the D site can not be sumoylated in absence of the C-terminus SUMO sites, we predicted a similar outcome for the short isoform sumoylation (c1/A). While, the short isoform has no extra C-terminus peptide and harbours only the D site, we detected a very weak sumoylated band for c1/A in IP. The specific folding of c1/A might make the D site competent enough to have a weak status of sumoylation, although not having any functional effect of its activity. Neither subcellular localization, as measured by immunofluorescence, nor the transctivation potential (reporter as well as RPA) seemed to be influenced by the weak sumoylation on c1/A.

**NFATc1/C sequestration into PML-nbs, an event which modulates NFATc1 function**

PML is localized to specific nuclear matrix associated subdomains, called PML-nb, which is the best characterized nuclear body in the human cell nucleus. (Dyck et al., 1994, Ascoli et al., 1991, Seeler et al., 1999). PML-nbs act as storage sites, modulating the concentration of nuclear proteins by sequestrating them at PML-bodies until required (Negorev et al., 2001). Recent findings established an important function of PML-nbs in modulating their residual protein function by protein-protein interactions as well as posttranslational modifications. In line with other previous observations, we have established that the sumoylation partitioned NFATc1 into PML-nbs. PML-nbs are the “house” of various kinds of protein factors, which are involved in gene regulation, DNA repair, tumour suppression, apoptosis etc. Therefore, a protein, which is targeted to PML-nbs, has a greater degree of probability for functional modulation by simply interacting with some of the residual proteins of the PML-nbs. In our study, we found that upon sumoylation NFATc1 interacts with the transcriptional repressors HDACs. Since sumoylation directs NFATc1 into PML-nbs and the PML-nbs harbor HDACs, it suggests that the PML-nbs

are the site for interaction between SUMO-modified NFATc1 and HDACs. In most of our IP experiments, when the ΔE mutant was introduced in IP, a significant reduction, but not complete loss of HDAC-NFATc1 interaction was observed. This raises the question if NFATc1 interaction with HDACs depends exclusively on sumoylation. It is possible that the SUMO tag might serves as a platform on NFATc1 to transiently interact with the members of repressor complex such as with mSin3a, NuRD or SMART/Ncor complexes in which HDACs have been identified (Downes et al., 2000). In this way, NFATc1 might get access to HDACs to interact. Mutating SUMO- consensus motifs from NFATc1 might disrupt this complex to a great extent, thereby reducing the strength of NFATc1 and HDACs interaction, which is reciprocally related to NFATc1 function. Because NFATc1 interacts with HDACs upon sumoylation, we argued that this event might lead to deacetylation of its target gene promoter, therefore inducing transcriptionally inactive chromatin. All of this ultimately leads to a decreased level of endogenous IL-2 production. Our argument was supported by experimental data data obtained from ChIP assays of the IL-2 promoter, which showed a significant reduction in histone H3 acetylation level (a transcriptionally active chromatin mark) upon NFATc1 sumoylation. The downstream effect of this event exhibited a similar trend resembling in the reduction of IL-2 mRNA as well as IL-2 protein levels. Thus, this observation established a new mechanism by which NFATc1 modulates chromatin architecture upon sumoylation.

NFATc1 regulates many other cytokine genes. Surprisingly, we did not observe a similar negative effect of NFATc1 sumoylation on other targets such as for lymphokines synthesized in Th1 and Th2 cells. In contrast, our RPA data showed a higher level of IFN-γ and IL-13 mRNAs, when NFATc1 has ensumoylated. This could be due to for the fact that sumoylation creates a promoter-specific transcriptome profile which determines the transcription of that particular NFATc1 target gene. When we fused SUMO1 protein to c1/C, such a factor showed a further strong increase in IFN-γ and IL-13 mRNA. Most interestingly, fusing SUMO-1 in front of ΔE mutant resulted in a loss of IL-13 mRNA, suggesting that not only the extent of sumoylation, but also the position of SUMO conjugation is important for the final outcome of NFATc1 activity. In summary, the functional effect of SUMO conjugation of NFATc1 is not universal for all NFATc1 target genes. The fine balancing act of this event may lie in the extent and the site of

sumoylation on NFATc1. In the context of IL-2 gene transcription, sumoylation has a negative impact on NFATc1 function. We have also shown that this is due to the action of HDACs on the acetylation pattern of IL-2 promoter. In other words, HDAC leaves an imprint for transcriptionally inactive chromatin. Histone H3 lysine 9 trimethylation (H3K9 m3) is one of the best characterized marks of closed chromatin or pericentric heterochromatin. We hypothesized that the sumo-modified NFATc1, which is transcriptionally repressive, might be associated with pericentric heterochromatin. Indeed, we found a higher association of SUMO-modified NFATc1 with heterochromatin compared to non-sumoylated NFATc1 protein. Because of sumoylated NFATc1 co-localization with PML-nbs as well as heterochromatin, it is tempting to interpret that SUMO-modified NFATc1 drags

IL-2 chromatin into the PML-nb and alters its epigenetic status to heterochromatin. However, we didn't observe any co-localization of PML-nbs and H3K9 m3 marks (data not shown). Therefore, It is likely that sumoylated NFATc1 recruits a repressosome within PML-nb, which then redistributes (having all the repressor complex) to the periphery or outside of PML-nb and promotes or associates with heterochromatin. This explanation falls in line with other previous observations where HP1, which binds to H3K9 m3 marks of heterochromatin, was shown to reside within PML-nbs (Sheeler et al., 1998), as well as to shuttle in-and-out of the PML-nb in order to induce heterochromatin (Luciani et al., 2006) formation. Other observations established that the chromatin of major histocompatibility complex-I (MHC-I) is associated with PML-nb, which controls MHC-I gene transcription (Shiels et al., 2001). Alltogether, sumoylation redirects NFATc1 into transcriptionally repressive parts of chromatin.

**SUMO-modification of NFATc1 and its co-localization with "transcription factories"**

Because of the higher transcriptional activity of c1/A and of ΔE mutant, it was obvious to hypothesize that the nuclear dots formed by them might co-localize with the areas of higher transcriptional activity. In the nucleus, numerous discrete spots have been identified which contain RNA polymerase II (RNA pol II or pol II), harbouring a higher degree of active transcriptional processes. Collectively, these spots are called "transcription factories" or pol II bodies (Jackson et. al., 1993, Iborra et. al., 1996, Grande et. al., 1997, Jackson et. al., 1998). Accordingly, as demonstrated by pol II colocalization, active c1/A and ΔE mutant associated with such transcription factories thereby getting access to the transcription machinery. Confirming the high degree of heterochromatin colocalization, SUMO-modification excludes NFATc1 from transcriptional hotspots.

**Concluding remarks**

Although another group has shown a similar kind of sumolyation for NFATc2 (Terui y. et. al., 2004), our data, for the first time, established an entirely different kind of functional regulation of NFAT namely NFATc1 by SUMO modification. Taken together, we have added a new mechanism by which NFATc1 function is repressed. Here, we have also established a functional specificity, which exists between the different NFATc1 isoforms, which is guided by sumoylation. However, the details of signaling pathway which might specifiy NFATc1 sumoylation in primary T cells is not clear to us. In immunofluorescence studies, it appeared that the colocalization of NFATc1-SUMO body was more pronounced in nTregs, in comparison with $CD4^+$ T cells. Thus, sumoylation might support nTregs to acquire an anergic phenotype leading to suppression of IL-2 production. In Tregs, Foxp3 orchestrates gene regulation by cooperating with NFATs (Wu y. et. al., 2006). Sumoylation of NFATc1 might acts as a mediator in this event. Finally, It will be very interesting to see if over-expressing sumo-modified NFATc1 in $CD4^+$ T cells gives rise to an anergic phenotype or if over-expressing ΔE mutant in nTregs trans-differentiates them to IL-2 producing T cells.

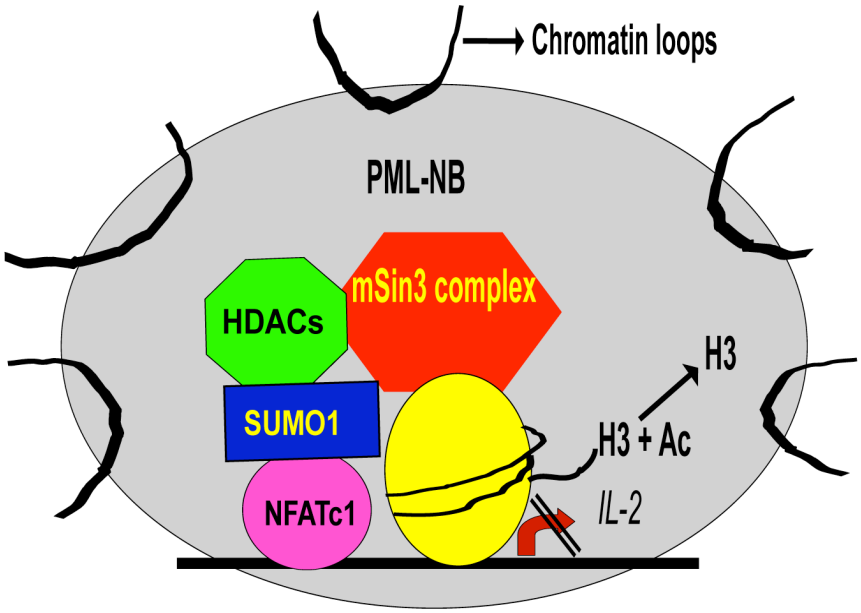



**Figure 4.1 Model showing the functional modulation of NFATc1 by sumoylation.** NFATc1 is sequestered into PML-NB upon SUMO modification where it interacts and recruits HDAC on *IL-2* promoter and induce deacetylation of histone (H3), making the IL-2 promoter transcriptionally inactive. The overall effect is negative on NFATc1 activity, which is manifested by downregulation of IL-2 production.

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# ABBREVIATIONS

| | |
|---|---|
| aa | Amino acid |
| Ab | Antibody |
| APC | Antigen presenting cell |
| ARF | Auxin Response Factor |
| $CaCl_2$ | Calcium chloride |
| CBP | CREB binding protein |
| CD | Cluster of differentiation |
| ChIP | Chromatin Immunoprecipiation |
| CK-1 | Casein kinase-1 |
| CSA | Cyclosporin A |
| ELISA | Enzyme likend immunosorbent assay |
| EMSA | Electrophoretic mobility shift assay |
| ER | Estrogen receptor |
| GFP | Green fluorescence protein |
| GSK3 | Glycogen synthase kinase 3 |
| HA | Hemagglutinin |
| HDAC | Histone deacetylase |
| HEK | Human embryonic kidney |
| H3K9 m3 | Histone H3 lysine 9 trimethylated |
| HP1 | Heterochromatin protein 1 |
| HSF1 | Heat shock protein 1 |
| IFN | Interferon |
| IgG | Immunoglobuling G |
| IκB | Inhibitor of NF-κB |
| IL | Interleukin |
| LiCl | Lithium chloride |
| MAPK | Mitogen activated protein kinase |

| | |
|---|---|
| MAR | Matrix attachment region |
| MCAF1 | MBD1-containing chromatin-associated factor 1 (MCAF1) |
| Ncor | Nuclear receptor co-repressor 1 |
| MDB1 | Methyl-CpG binding domain protein 1 |
| MEF | Myeloid elf-1-like factor |
| $Mgcl_2$ | Magnesium chloride |
| ND10 | Nuclear domain 10 |
| NEM | N-ethylmaleimide |
| NES | Nuclear export sequence |
| NLS | Nuclear localization sequence |
| NFAT | Nuclear factor of activated of T cells |
| NF-κB | Nuclear factor κB |
| NuRD | Nucleosomal remodeling and histone deacetylase |
| PCR | Polymerase chain reaction |
| PIAS1 | Protein inhibitor of activated STAT1 |
| PML-nbs | PML nuclear bodies |
| POD | PML oncogenic domain (POD) |
| RAG | Recombinase activating gene |
| RanGAP1 | Ran GTAase activating protein 1 |
| RanBP2 | Ran binding protein 2 |
| RLU | Relative luciferase unit |
| RPA | RNase protection assay |
| SENP | Sentrin-specific protease |
| SUMO | Small ubiquitin like modifier |
| TAD | Transactivation domain |
| TPA | 12-O-tetradecanoyl-phorbol-13-acetate |
| TCR | T cell receptor |
| TDG | Thymine DNA glycosylase |
| Th | T helper cell |

| | |
|---|---|
| T/I | TPA/Ionomycine |
| Tm | Tamoxifen |
| YFP | Yellow fluorescence protein |

# CURRICULUM VITAE

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**Nayak A**, Gloeckner J, Novak E, Serfling E, Berberich-Siebelt F, SUMOylation suppresses NFATc1 transcriptional activity by sequestration into nuclear bodies in T cells. Oral and poster presentation *in* ***EMBO conference on Chromatin and Epigenetics ( May 2007)***

**Nayak A**, Gloeckner J, Novak E, Serfling E, Berberich-Siebelt F, Functional consequences of NFATc1 SUMOylation in T cells. Poter presentation at ***7th EMBL Transcription meeting (2006***).

## List of Publications:

**Arnab Nayak**, Judith Glöckner-Pagel, Mathias Buttmann, Tobias Bopp, Edgar Serfling, and Friederike Berberich-Siebelt. Sumoylation of the transcription factor NFATc1 leads to its subnuclear relocalization and IL-2 repression by HDAC. *(Submitted)*

Doris Schmidt, Friederike Berberich-Siebelt, **Arnab Nayak**, Anneliese Schimpl and Ingolf Berberich. Blimp-1Δexon7: A naturally occurring Blimp-1 deletion mutant with auto-regulatory potential. *(Submitted)*

Hiraoki Azukizawa, Nobuo Kanazawa, **Arnab Nayak**, Friederike Berberich-Siebelt, and Manfred B. Lutz. Peripheral de novo induction of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells by steady state migratory dendritic cells. *(Manuscript in preparation)*

Eisaku Kondo, Rene Rost, Chen Wen, **Arnab Nayak**, Friederike Berberich-Siebelt, Sergei Chuvpilo, Stefan Klein-Hessling, Astrid Elter, Lars Nitschke, Andris Avot, and Edgar Serfling. The crosstalk between NFATc1 and NF-κB Transcription Factors Protects B-Lymphocytes against Apoptosis. *(Manuscript in preparation)*

## Declaration

I hereby declare that the submitted dissertation was completed by myself and none other, and I have not used any sources or materials other than those enclosed. Moreover, I declare that the following dissertation has not been submitted further in this form and has not been used for obtaining any other equivalent qualification in any other organization. Additionally, other than this degree I have not applied or will not attempt to apply for any other degree, title or qualification in relation to this work.

Arnab Nayak