Regulation of NFATc3 stability by SUMO and E3 ubiquitin-ligases Trim39 and Trim17
Meenakshi Basu Shrivastava

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Le 14/12/2020
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Examinateur
Dedicated to my loving parents, Mrs. Malika Basu and Mr. Tarun Kanti Basu

&

to the love of my life, my husband, Dr. Aatmesh Shrivastava
 Acknowledgements

My journey to France commenced in 2015 and five years later, I really feel very fortunate for being able to document my experience, both at scientific and human level.

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**Abbreviations**

**A**

AD  Alzheimer’s Disease  
ADAR  Adenosine Deaminase that acts on RNA  
AL  Autophagy Lysosome  
AP  Activator Protein  
Apafl  Apoptotic Protease Activating Factor 1  
APL  Acute Promyelocytic Leukaemia  
APP  Amyloid Precursor Protein  
APR  Acute Promyelocytic Leukaemia  
ARF  ADP-ribosylation Factor  
ATG  Autophagy-related Genes/Proteins  
ATO  Arsenic Trioxide  
Az  Antizyme  
Aβ  Amyloid beta

**B**

BCL  B-cell Lymphoma  
BD  Behcet’s Disease  
BDNF  Brain-derived Neurotrophic Factor  
Bdnf  Brain Derived Nerve Growth Factor  
BHK  Baby Hamster Kidney  
BR  Bromodomain  
BRAT  BRAin Tumour  
bZIP  Basic Region-leucine Zipper

**C**

Cactin  Cactus-interactor  
CaN  Calcineurin  
CASP  Caspase  
CBP  CREB-binding Protein  
CC  Coiled-coil domain  
Cdc34  Cell Division Cycle 34  
CDK  Cyclin Dependent Kinases  
CGN  Cerebellar Granule Neurons  
CHIP  C-terminus of HSP70-interaction Protein  
CHX  Cycloheximide  
CK  Casein Kinase  
CK2  Casein Kinase2  
CKI  Cyclin-dependent Kinase Inhibitor  
C-L  Caspase-like
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>CLE</td>
<td>Cutaneous Lupus Erythematosus</td>
</tr>
<tr>
<td>CNRS</td>
<td>Center National de la Recherche</td>
</tr>
<tr>
<td>COS</td>
<td>C-terminal subgroup One Signature</td>
</tr>
<tr>
<td>COST</td>
<td>European Cooperation in Science and Technology</td>
</tr>
<tr>
<td>CP</td>
<td>Core Particle</td>
</tr>
<tr>
<td>CREBBP</td>
<td>CREB-binding Protein</td>
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<td>CTD</td>
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<td>Chymotrypsin-like</td>
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<td>Dual-specificity Tyrosine-phosphorylation Regulated Kinase 1a</td>
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<td>EFP</td>
<td>Oestrogen-responsive Finger Protein</td>
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<td>ERK</td>
<td>Extracellular Signal Related Kinase</td>
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<td>FIL</td>
<td>Filamin-type Immunoglobulin</td>
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<td>FN</td>
<td>Fibronectin type</td>
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<td>GABA</td>
<td>Gamma-aminobutyric Acid</td>
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<td>Growth Associated Protein Gene</td>
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<td>Hydrophobic Cluster SUMOylation Motif</td>
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<td>HNSCC</td>
<td>Human Head and Neck Squamous cell Carcinoma</td>
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<td>Matrix Metalloproteinases</td>
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<td>MOAP</td>
<td>Modulator of Apoptosis</td>
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<tr>
<td>MOMP</td>
<td>Mitochondrial Outer Membrane Permeabilization</td>
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<tr>
<td>MPN</td>
<td>Mpr1-Pad1 N Terminal</td>
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<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
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<tr>
<td>MT</td>
<td>Mitochondria</td>
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<tr>
<td>Abbreviation</td>
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<td>-----------</td>
</tr>
<tr>
<td>NB</td>
<td>Nuclear Bodies</td>
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<td>NCoR</td>
<td>Nuclear Receptor co-Repressor</td>
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<tr>
<td>NDSM</td>
<td>Negatively Charged Amino-acid-dependent SUMOylation Motif</td>
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<td>Nedd8</td>
<td>neural precursor cell expressed, developmentally downregulated gene 8</td>
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<td>NEM</td>
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<td>NFAT</td>
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<td>NLS</td>
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<td>OTUs</td>
<td>Ovarian Tumor Protease</td>
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<tr>
<td>PBL</td>
<td>Peripheral Blood Lymphocyte</td>
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<tr>
<td>PCD</td>
<td>Programmed Cell Death</td>
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<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
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<td>Parkinson’s Disease</td>
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<tr>
<td>PHD</td>
<td>Plant Homeodomain</td>
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<tr>
<td>PIAS</td>
<td>Proteins Inhibitor of STAT</td>
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<tr>
<td>PINIT</td>
<td>Pro-Ile-Asn-Ile-Thr</td>
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<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-Bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
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<td>PKB</td>
<td>Protein Kinase B</td>
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<td>PLA</td>
<td>Proximity Ligation Assay</td>
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<td>PLCγ</td>
<td>Phospholipase Cγ</td>
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<td>Phorbol12-myristate 13-acetate</td>
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<td>PMA</td>
<td>Phorbol-myristate Acetate</td>
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<td>ProCASP9</td>
<td>Procaspase-9</td>
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<td>PSDM</td>
<td>Phosphorylation-dependent SUMOylation Motif</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational Modification</td>
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**R**

RA Rheumatoid Arthritis
RanBP2 Ran-binding protein 2
RanGAP RanGTPase-activating protein
RANKL Receptor Activator of Nuclear factor κB Ligand
RARα Retinoic Acid Receptor
RBP RNA Binding Proteins
RFP RET Finger Protein
RHR Rel-Homology Region
RIG Retinoic Acid-inducible Gene
RING Really Interesting New Gene
RNF111 RING finger protein Arkadia
ROS Reactive Oxygen Species
RP Regulatory Protein
Rpn Regulatory particle non-ATPase
RPP21 Ribonuclease P/ MR P 21kDa
Rpt Regulatory particle triple AAA-ATPase
RT-PCR Reverse-transcription Polymerase Chain Reaction

**S**

S/T Serine/threonine-rich C-terminal
SAP Scaffold attachment factor-A/B, Acinus, and PIAS
SCM SUMO Consensus Motif
SIMs SUMO Interacting Motifs
SLE Systemic Lupus Erythematosus
SN Substantia Nigra
SNP Single Nucleotide Polymorphisms
SN Single Nucleotide Polymorphisms
SNURF Small Nuclear RING Finger Protein
SOC Store-operated Calcium Channels
SOCE Store-operated Ca2+ Entry
SP-CTD Unique C-terminal Domain
SS Sjogren’s Syndrome
STUbLs SUMO-targeted Ubiquitin Ligases
SUMO Small Ubiquitin-like Modifier

**T**

TCR T-Cell Receptor
terf testis RING Finger Protein
TF Transcription Factors
TFP Testis Abundant Finger Protein
Th T-helper
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>TIF</td>
<td>Transcriptional Intermediary Factor</td>
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<tr>
<td>T-L</td>
<td>Trypsin-like</td>
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<tr>
<td>TM</td>
<td>Transmembrane</td>
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<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
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<td>TRIM</td>
<td>TRIPartite Motif</td>
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<td>Transfer RNA</td>
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<td>UBA</td>
<td>Ubiquitin-Associated Domain</td>
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<td>Uba1</td>
<td>Ubiquitin-like Modifier-activating Enzyme</td>
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<td>Ubc1</td>
<td>Ubiquitin-conjugating Enzyme E21</td>
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<td>USPL</td>
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<tr>
<td>VHL</td>
<td>Von Hippel-Lindau tumor suppressor</td>
</tr>
<tr>
<td>VWA</td>
<td>Von Willebrand factor A</td>
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<td>WBS</td>
<td>Williams-Beuren Syndrome</td>
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<td>ZOL</td>
<td>Zoldronic Acid</td>
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Chapter 1  The Ubiquitin-Proteasome System

The ubiquitin-proteasome system is a major protein degradation system after the lysosomal system in eukaryotes (Grune et al. 2001; Berke and Paulson 2003; Goldberg 2003; Levine and Klionsky 2004; Glickman and Ciechanover 2002; Schwartz and Ciechanover 2009). Once the proteins are conjugated to ubiquitin chains, they are directed to a macromolecular protease known as the 26S proteasome where they are eventually degraded (Kleiger and Mayor 2014; Glickman and Ciechanover 2002; Bard et al. 2018; Collins and Goldberg 2017). The entire journey from transfer of ubiquitin onto the protein to the final degradation of the protein into peptides involves lot of complex processes. Indeed, it is safe to say that we have come a long way in the field of ubiquitin research since the discovery of ubiquitin in 1975 (Goldstein et al. 1975).

1.1  Ubiquitin and the Enzymatic Cascade

1.1.1  Ubiquitin

Ubiquitin is a 76 amino acid protein with a molecular weight of 8.5kDa. It is highly conserved, from yeast to man, implying that many of its surfaces are recognized by the UBDs (Ubiquitin binding domains). In mammals, ubiquitin is encoded by four different genes. The genes, UBA52 and RPS27A, code for a single copy of ubiquitin fused to the ribosomal proteins L40 and S27A, respectively. The other two genes namely, UBB and UBC genes code for poly-ubiquitin precursor proteins (Kimura and Tanaka 2010).

The hydrophobic surface comprising of Isoleucine44 (Ile44), Leucine8 (Leu8), Valine70 (Val70), and Histidine68 (His68) facilitates the recognition of ubiquitin by the proteasome (Dikic, Wakatsuki, and Walters 2009). Interestingly, the HECT E3’s (a class of E3 ubiquitin ligases), DUBs (deubiquitinases), and UBDs can recognize another hydrophobic patch, Ileucine36 (Ile36) which involves Leu77 and Leu73 of the ubiquitin tail and can mediate interactions between ubiquitin molecules in chains (Komander and Rape 2012). Ubiquitin can be further modified by either ubiquitin itself or other post-translational modifications such as small ubiquitin-like modifier (SUMO), phosphorylation and acetylation (Swatek and Komander 2016).

1.1.2  Enzymes at work

The ubiquitination of proteins involves sequential action of three ubiquitin enzymes namely E1 activating enzyme, E2 conjugating enzyme, and E3 ubiquitin ligase. An E1 activating enzyme
activates ubiquitin in an ATP-dependent manner by forming a covalent bond between the C-terminal end of ubiquitin and a cysteine residue in its active site (Figure 1).

![Figure 1: Structure of ubiquitin](image1.png)

The thioesterified ubiquitin is then passed to the E2 conjugating enzyme (Figure 2A). The final step involves the E3 ubiquitin ligase that binds to both the E2-bound ubiquitin and the substrate, therefore promoting the transfer of ubiquitin onto the substrate (Figure 2C) (Bence, Sampat, and Kopito 2001; Adams 2003; B. Gong et al. 2016). The mammalian ubiquitin cascade consists of two members of the E1 family which tags all the E2s with ubiquitin whereas there are 40 E2s that help facilitate the delivery of ubiquitin to more than 600 E3s (Deshaiies and Joazeiro 2009).

Proteins, in general, are ubiquitinated on the lysine residues which results in the formation of an isopeptide bond between the carboxyl end of ubiquitin and the lysine primary amine. Proteins can be

![Figure 2: Schematic representation of the three steps involved in ubiquitination of a substrate.](image2.png)
either mono-ubiquitinated (conjugated to one ubiquitin monomer) or polyubiquitinated (conjugated to several ubiquitins). The state of protein ubiquitination (mono or poly-ubiquitinated) and the type of ubiquitin chain attached on a protein finally decides its fate i.e. whether it undergoes degradation or participates in cellular responses (Komander and Rape 2012; P. Xu et al. 2009; Tokunaga et al. 2009). To make matters more interesting, ubiquitin can crosstalk with other ubiquitin-like modifiers (Ubl’s) such as SUMO, Nedd8 (neural precursor cell expressed, developmentally downregulated gene 8), phosphorylation (Schulman and Harper 2009).

**E1 Activating Enzyme**

The multidomain E1 enzymes aid in the activation of ubiquitin and thus facilitate its transfer to the active site of E2. This is a crucial function of E1s to maintain cellular homeostasis because failure to do so results in the entire UPS being shutdown (Yili Yang et al. 2007). Several structural studies have contributed to our understanding of how E1 activates and transfers ubiquitin to the E2. The E1-Uba1 (ubiquitin-like modifier-activating enzyme) for ubiquitin is a monomeric protein of 110-120kDa (I. Lee and Schindelin 2008; Lake et al. 2001; S. K. Olsen and Lima 2013). The E1-mediated reaction involves ATP-dependent adenylation of the C-terminal carboxyl group of ubiquitin, the formation of a thioester bond between the catalytic cysteine residue of E1 and the C-terminus of ubiquitin, and ultimately the transfer of ubiquitin onto the catalytic cysteine of E2. An elegant study presented the crystal structure of a UbE1-E2 (Ubc4)/Ub/ATP.Mg complex stabilised by inducing disulfide bond between the E1 and E2 active sites. It revealed that the conformational changes in the E1 brings E1 and E2 active sites together (S. K. Olsen and Lima 2013).

**E2 conjugating enzymes**

The transfer of ubiquitin to the substrates is catalyzed by the E2 conjugating enzymes (Pickart 2001; Ye and Rape 2009; Varshavsky 2012; Ciechanover 2015; Stewart et al. 2016). All E2s share an approximately 150 amino acid conserved core domain. The thioester bond between the C-terminal end of ubiquitin and the conserved cysteine residue of the E2 forms the core of the E2 active site. The positioning of the thioester bond in an optimal conformation governs the rate of ubiquitin transfer and this could be achieved by the E2, the E3 or their combination. Initial study had shown that E2 Ubc1 (ubiquitin-conjugating enzyme E21) forms a non-covalent interface with a ubiquitin whose active site is thioesterified (Hamilton et al. 2001). On the other hand, it was also demonstrated that in addition to the covalent thioester bond, E2s Cdc34 (cell division cycle 34) and Ube2S (ubiquitin-conjugating enzyme E2S), also form noncovalent interfaces with ubiquitin (Saha et al. 2011; Wickliffe et al. 2011). This suggested that E2s catalyze ubiquitin transfer (in part) by holding ubiquitin against an interface on the surface of E2 which optimizes the thioester bond position in the active site. Despite the fact that E2s have similarity, different E2s exert different biological functions. For example, many substrates associated with cell cycle regulation (such as cyclin-dependent-kinase inhibitor Sic1 and the G1 cyclins) were stabilised with mutations in the Cdc34 gene alone. The stabilization of these two substrates reflects the specificity of Cdc34 when it partners with two different SCF E3s (Pickart 2001).
**E3 ubiquitin-ligases**

Currently there are more than 600 E3s in humans which participate in the ubiquitination pathway by mediating substrate specificity. Based on the presence of characteristic domains and the mechanism by which they transfer ubiquitin on to the substrate, E3 ubiquitin-ligases can be classified into three types: RING E3s, HECT E3s, and RBR E3s (Morreale and Walden 2016).

RING E3s: They are the most abundant type of ubiquitin ligases which are characterized by the presence of a zinc-binding domain called Really Interesting New gene or by a U-box domain, which has RING domain but lacks the presence of zinc. The RING and U-Box domains are responsible for binding the ubiquitin-charged E2 and stimulating ubiquitin transfer. The RING E3s mediate a direct transfer of ubiquitin to the substrate thereby acting as a scaffold to orient the ubiquitin-charged E2 with respect to substrate protein. The RING E3s could exist as either monomers, homodimers or heterodimers while the U-Box domains can function as monomers or homodimers. Examples of RING E3s include Cullin-RING ligases (CRLs) and Anaphase promoting complex/cyclosome (APC/C) (Deshaies and Joazeiro 2009). Interestingly, RING E3s can be regulated by neddylation, phosphorylation, and interaction with small molecules.

HECT E3s: HECT (Homologous to the E6 AP carboxyl terminus) domain family of E3 ligases catalyze the ubiquitin transfer to the substrate protein in a two-step reaction: ubiquitin is transferred from a catalytic cysteine to the E3 and then from E3 to the substrate. Located at the C terminus of the proteins, the conserved HECT domain is characterized by a bi-lobar architecture where the N-terminal lobe interacts with the ubiquitin charged E2 whereas the C-terminus contains the catalytic cysteine. Human HECTs can further be classified into three sub-families: (i) Nedd4 family which contains tryptophan-tryptophan (WW) motifs (ii) HERC (HECT and RCC-1-like domain) family which contains one or more regulators of chromosome condensation1(RCC1)-like domains (RCDs), and (iii) other HECTs that contain various domains (Metzger, Hristova, and Weissman 2012; Morreale and Walden 2016). Furthermore, the intramolecular interactions facilitate the regulation of catalytic activity of HECT E3s that keep the protein in an autoinhibited state and this autoinhibitory state is released in response to various signals.

RBR E3s: The RING-between RING-RING (RBR) E3s catalyze the ubiquitin transfer via a two-step reaction where ubiquitin is transferred to a catalytic cysteine on the E3 first and then to the substrate. These E3s contain two predicted RING domains (RING1- and RING-2) separated by an in-between-RING domain (IBR). RING1 recruits ubiquitin charged E2 while RING domain possesses the catalytic cysteine and is referred to as Rcat (required-for-catalysis) domain. Similar to the RING2 domain, the IBR domain lacks the catalytic cysteine and is referred to as BRcat (benign-catalytic) domain. Several domains in RBRs are involved in intramolecular interactions that keep the protein in an auto inhibited state and again, this state is released through mechanisms that include phosphorylation or protein-protein interactions (Dove and Klevit 2017; Morreale and Walden 2016).
1.1.3 Deubiquitinating enzymes (DUBs) - spoilers alert

The recycling of ubiquitins, once the substrate has been committed to the degradation pathway, is critical for maintaining ubiquitin homeostasis. As a result, both the proteasomes and the lysosomal sorting machinery are associated with deubiquitinating enzymes (DUBs) (Metzger, Hristova, and Weissman 2012; Morreale and Walden 2016). DUBs greatly influence many biological processes and cellular pathways such as tumorigenesis, DNA damage response, and DNA repair pathway to name a few. DUBs catalytically cleave single Ub or polyubiquitin chains from proteins and thus help in maintaining the ubiquitin pool. The human genome encodes approximately 100 DUBs and can be classified into six families. Ubiquitin-specific proteases (USP) family is the largest family boasting approximately 55 members. Other DUBs belong to ubiquitin C-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Josephin, JAB1/MPN/MOV34 (JAMMs), and motif interacting with Ub-containing novel DUBs (MINDY)s families (Komander, Clague, and Urbé 2009; Nijman et al. 2005; Turcu, Ventii, and Wilkinson 2009; He et al. 2016).

The main roles of DUBs include modulating the activity of E2s, counteracting E3s, assisting degradation machinery, maintaining ubiquitin homeostasis, etc. DUBs are capable of inhibiting ubiquitination by interfering with the reactivity and the formation of the E2-Ub intermediates. For example, USP7 (DUB found in all eukaryotes) removes the ubiquitin from target proteins such as Mdm2, ICPO, and p53 (Nicholson and Suresh Kumar 2011). USP7 binds to the N-terminal ASTS sequence of UbE2E1 (an E2) and forms a complex. This
binding results in the attenuation of UbE2E1-mediated ubiquitination via the ASTS motif within its N-terminal extension and the catalytic domain of USP7. Interestingly, the disruption or inactivation of the interaction between USP7 and UbE2E1 leads to the destabilization of UbE2E1 (Sarkari et al. 2013). DUBs often co-regulate with E3 ligase partner to orchestrate the ubiquitin loading and removal in target proteins. For example, the stability of p53 is regulated by the DUB, USP10, under the physiological conditions as well as in DNA damage response with its E3 partner Mdm2. USP10 is responsible for maintaining stable levels of p53 in the cytosol, however post DNA damage, part of USP10 translocates to the nucleus to deubiquitinate p53. This translocation ultimately results in a boost of p53 activation (J. Yuan et al. 2010).

DUBs assist the degradation machinery (Figure 4). The hydrolysis of ub-chains before proteins are unfolded and degraded (Verma et al. 2002) is carried out by the DUB, POH1. It is known that two other DUBs, Ubp6/USP14 and UCH37, oppose protein degradation as they trim ubiquitin chains from the distal end of the chain. This results in a decreased affinity of the protein for the proteasome (Jacobson et al. 2014; Bashore et al. 2015). DUBs maintain the ubiquitin homeostasis by generating Ub precursors from encoded genes, trimming the Ub precursors to free Ubs, disassembling polyubiquitin chains from proteins, and by recovering ubiquitin from chains. The major DUB, USP5, releases ubiquitin from unanchored isopeptide-linked ubiquitin chains (Reyes-Turcu et al. 2006).

An Insight into Ubiquitin Chain-Signalling

The nature of the ubiquitin modification governs the fate of the ubiquitinated substrate. Ubiquitin plays an integral role in modulating functions of various proteins and its dysregulation has a deep

Figure 4: The figure displays a small list of E2 or E3 enzymes that help in the assembly and the DUBs that support the disassembly of different ubiquitin chains. The biological processes associated with different chain types are also depicted by a cartoon below. From (Swatek and Komander 2016)

1.1.4 An Insight into Ubiquitin Chain-Signalling

The nature of the ubiquitin modification governs the fate of the ubiquitinated substrate. Ubiquitin plays an integral role in modulating functions of various proteins and its dysregulation has a deep
impact in the development of numerous human diseases. It is well established that each ubiquitin chain type has a different function (Akutsu, Dikic, and Bremm 2016a). The covalent attachment of ubiquitin on the protein substrates can lead to either the elimination of the substrate by the proteasome or it might affect its localisation, substrate activity, binding with partners and can also have non-proteolytic consequences (Oh, Akopian, and Rape 2018).

A single ubiquitin molecule (mono or multi-mono) can modify one or multiple Lys (K) residues in proteins. In a ubiquitin chain, ubiquitin moieties can be conjugated through one of their K residues (K6, K11, K27, K29, K33, K48, K63) or N-terminal Met residue (M1) (Meyer and Rape 2014). There are two types of ubiquitin conjugates: the homotypic type where the same K or methionine residue connects all the building blocks of chains and heterotypic type which contains mixed ubiquitin chain linkages within the same polymer. The different polymers of ubiquitin on a substrate (homo and heterotypic) lead to distinct cellular responses. Additionally, there might be a possibility to form mixed and branched chains where the ubiquitin molecule is ubiquitinated at two or more than two sites (Figure 4) (Swatek and Komander 2016). Recent studies have also added new layers of complexity wherein ubiquitin can be modified by several ubiquitin-like modifications such as SUMOylation, acetylation, and phosphorylation (Swatek and Komander 2016; Akutsu, Dikic, and Bremm 2016b; Cuijpers, Willemstein, and Vertegaal 2017; Kane et al. 2014; Kazlauskaite et al. 2014; Koyano et al. 2014).

1.1.5 Complexity of Ubiquitin Chain Assembly

The study of ubiquitin chains is evolving at a rapid pace and varied approaches exist (Figure 5). The chain types that regulate tremendous amounts of biological processes can be like a spider’s web.
Monoubiquitination was thought to be involved in subcellular localization of proteins (van der Horst et al. 2006) rather than promoting degradation (Hicke 2001). The requirement of a minimum of four or more ubiquitin moieties for degradation is being challenged. For instance, Lu et al. showed that used single-molecule kinetics studies to demonstrate that monoubiquitination of cyclinB1 was able to stimulate degradation by human proteasomes (Lu et al. 2015). In another study by Dimova et al., it was shown in Xenopus extracts that multi monoubiquitination of CyclinB1 was an efficient signal for its degradation (Dimova et al. 2012).

If we talk about homotypic ubiquitin chains, they are formed when a substrate is modified by the same type of ubiquitin chain. It was identified that homotypic K11-polyubiquitin chains did not bind to the proteasome or to the ubiquitin receptors associated with the proteasome. Considering an example of Cyclin B as the substrate, it was shown that the homotypic K11 linkages did not signal its degradation. In contrast, heterotypic K11/K48-polyubiquitin chains were able to signal degradation by the proteasome (Swatek and Komander 2016).

The heterotypic (mixed) chains can be categorized into two groups: a. Tandem mixture of different linkages (mixed or hybrid chains) or b. A ubiquitin moiety within a polyubiquitinated chain can be attached to different linkages (branched or forked chains) (Figure 5). An example of cellular roles of mixed chains is the use of K11/K63 mixed linkages in intracellular trafficking. These are formed the process of endocytosis of major histocompatibility complex class I (MHC I) membrane proteins. The role of branched ubiquitin chains emerged with APC/C, an E3 ubiquitin ligase that regulates mitosis. A study highlighted that in-vitro branched chains comprising of K11/K48 (and also K11/K63) enhanced the proteasomal degradation of the substrates. They suggested that the density of the ubiquitin signal could be increased when K11-branched from already formed short K48-linked chains that may enable multiple K11-linked chains from a single K48-linked chain (Meyer and Rape 2014).

### 1.1.6 Mechanisms of the Chain Assembly

Over the years of the ubiquitin study, several models for chain assembly have been proposed (Hochstrasser 2006). The two basic mechanisms that are followed are the sequential addition and the en bloc transfer. The former involves the transfer of individual ubiquitin moiety onto the substrate. In the case of the latter, the pre-formed chains are transferred to the active cysteine site of an E2 or HECT/RBR E3 to a substrate (Deol, Lorenz, and Strieter 2019a).

#### Sequential Addition

According to this model, each ubiquitinated protein acts as a substrate to form long substrate-linked chains (Figure 6A). It, therefore, becomes necessary to perform kinetic studies in order to validate this mechanism as we should be able to detect individual products on rapid timescales ranging from milliseconds to seconds. This mechanism was supported by a study that employed a millisecond kinetics measurement approach (Pierce et al. 2009). Using Cdc34 as E2, SCFCDC4 and SCFβ-TRCP as a RING domain-containing E3 ubiquitin ligase and Cyclin E as a substrate, it was demonstrated that
each round of chain elongation used each reaction intermediate as a substrate. The study concluded that most enzyme-substrate encounters were not productive as the first ubiquitin transfer is the slowest step in the assembly of ubiquitin chains. After the first ubiquitin is successfully transferred, it becomes easier to sequentially add the ubiquitins on the growing chain before the dissociation of the enzyme-substrate complex. The rate of this sequential addition of ubiquitins ($k_{ub-n}$) is faster than substrate dissociation rate ($k_{off}$) resulting in processive poly-ubiquitination of the substrate. The consequence of sequential addition is that the fate of the ubiquitinated substrate would depend on the rate with which ubiquitin chains can be formed on it (Deol, Lorenz, and Strieter 2019a).

**En-Bloc Transfer**

In contrast to the above-mentioned model, there are studies indicating that, pre-formed chains are assembled on the active site of cysteine of an E2 or E3 before the transfer on the substrate (Figure 6B). En bloc transfer mechanism would be impeccable if we consider the efficiency with which a substrate could be poly-ubiquitinated. The advantage of this mechanism over the sequential addition is that in order for a substrate to be tagged with ubiquitin chains of sufficient length, the complex of E3-substrate need not be long-lived. On the other hand, one of the cons of this model is that the mechanism of these pre-formed chains should be faster than the substrate transfer.

Kinetic studies supporting this model were done using UBE2G2 (Fang et al. 2001; B. Chen et al. 2006), a human E2 and Uch7 (Biederer, Volkwein, and Sommer 1996; Hiller et al. 1996; Bays et al. 2001; Deak and Wolf 2001), its yeast ortholog and GP78, an endoplasmic reticulum residing RING E3 ubiquitin-ligase (W. Li et al. 2007). The mentioned E2, UBE2G2, is known to be associated with the ER membrane and it forms K48 linked-ubiquitin chains on the misfolded proteins which are exported from the ER during ERAD. UBE2G2 together with GP78 catalyzed the assembly of ubiquitin chains on the cysteine active site. The driving factor behind this pre-assembly process is the ability of GP78 to oligomerize. The hetero-oligomerization of UBE2G2 and GP78 can bring the
active sites of several UBE2G2 molecules into close proximity (W. Liu et al. 2014). Therefore, the en bloc assembly of ubiquitin chains by GP78 implies that the ubiquitin conjugated to the active site of GP78 provides the acceptor lysine residue (K48) to attack the C-terminus of another thioester-linked ubiquitin attached to either a GP78 subunit or an associated E2 (Deol, Lorenz, and Strieter 2019b).

1.2 26S Proteasome

Protein degradation is an indispensable process to assess the level of proteins in a cell and the maintenance of protein homeostasis. In mammalian cells, the major part of non-lysosomal protein degradation is the proteasome. The proteasome is a large complex protease that is responsible for the degradation of intracellular proteins. The 26S proteasome consists of two subcomplexes: 20S core particle, which is responsible for the proteolytic cleavage of the protein substrates and a 19S regulatory particle, which serves as a site for initiation of substrate recognition (Murata, Yashiroda, and Tanaka 2009; Collins and Goldberg 2017; Budenholzer et al. 2017). The polymerization of ubiquitin, the main molecule that works with the proteasome, serves as a signal for the degradation of numerous target proteins. Once the target proteins are tagged with polyubiquitin chains, they are shuttled to the proteasome where they are proteolytically broken down. Almost all basic cellular processes such as cell cycle progression, apoptosis, signal transduction, immune responses are controlled by the ubiquitin-proteasome system (UPS) (Groothuis et al. 2006; Sujashvili 2016; Grumati and Dikic 2018; Michael James Emanuele and Enrico 2019; Kliza and Husnjak 2020; Q. Fan et al. 2020).

Figure 7: Schematic representation of the 26S proteasome. a. The 26S consists of the catalytic 20S proteasome (CP) and the 19S regulatory particle (RP); b. Composition of the 26S proteasome: 19S RP (composed of lid and base subunits) and the 20S CP (composed of two outer α-rings and two inner β-rings). From (Murata, Yashiroda, and Tanaka 2009).
1.2.1 Components of the 26S Proteasome and its Assembly

20S Core Particle (CP)
In eukaryotes, the 20S core particle (CP) forms a cylinder-like structure composed of 2 outer α rings and 2 inner β rings. Indeed, it is a packed particle constituting 7 structurally similar α and β subunits arranged in the order: \( \alpha_1 \cdot \beta_1 \cdot \alpha_1 \cdot \beta_1 \cdot \alpha_1 \). The alpha rings form the substrate entry channel whereas proteolytic cleavage takes place in the beta rings. The α subunits in the CP include conserved N terminal peptide extensions. These are important as they form a gate controlling the substrate entry via the central α ring channels. The crystal structure of the α-ring suggests that the center of the α-ring is completely closed. This closed conformation prevents the proteins from entering into the β ring (Arendt and Hochstrasser 1997; W. Heinemeyer, Ramos, and Dohmen 2004; Murata, Yashiroda, and Tanaka 2009).

The three main proteolytic active sites in the two β-rings (β1, β2, β5) contain catalytically active threonine residues at its N terminus indicating that proteasome is a threonine protease. The β1 is associated with caspase-like, β2 with trypsin-like and β5 with chymotrypsin-like activities that have the ability to cleave peptide bonds at the C terminus of acidic, basic and hydrophobic amino acid residues, respectively (Groll et al. 1997; Unno et al. 2002; Tanaka 2009). The protein substrates are cleaved into oligopeptides that range from 3 to 15 amino acid residues by the 20S proteasome. They are then subsequently hydrolyzed to amino acids by oligo-peptidases or amino-carboxyl peptidases.

19S Regulatory Protein (RP)
The 19S regulatory protein (RP), also known as proteasome activator (PA700), generally caps the proteasome on either or both the ends of the central 20S CP. The 19S RP recognizes the protein substrates followed by removal of ubiquitin chains, unfolds the protein substrates and then translocates them to the 20S CP for proteolytic cleavage. The process of translocation requires the hydrolysis of ATP, which is achieved by a heterohexameric ring of ATPases present in the RP. The 19S RP is composed of two conformationally dynamic sub-complexes: a 9-subunit lid and a 9-subunit base (Figure 7) (Tanaka 2009; Finley 2009; Im and Chung 2016; Saeki 2017).

The Lid Subcomplex
The lid subcomplex is composed of at least 9 Regulatory particle non-ATPase (Rpn) subunits: Rpn3, 5, 6, 7, 8, 9, 11, 12 and 15 (Figure 7) (Tanaka 2009). The key function of the lid is to deubiquitinate the captured substrates. Indeed, it is the Rpn11 subunit of the lid that functions to recycle the ubiquitin and is the principal component of the lid subcomplex. Rpn11 is a zinc \((\text{Zn}^{2+})\) dependent deubiquitinase (DUB) of the Mpr1-Pad1 N terminal (MPN) family and is in charge of removing the ubiquitin chains attached to the substrates before they enter the AAA+ATPase ring (located at the center of the base). Additionally, in mammalian cells, two other DUBs: Usp14 (associated with Rpn1 of the base subcomplex) and Uch37 (associated with Rpn13 of the base subcomplex) cleave the
ubiquitin at a distal site (Finley 2009; de Poot, Tian, and Finley 2017). The function of other lid subunits needs to be further elucidated.

The Base Subcomplex

The base subcomplex contains 6 homologous regulatory particle triple AAA-ATPase subunits (Rpt1-6) and 4 non-ATPase subunits (Rpn1, 2, 10 and 13) (Figure 7) (Tanaka 2009). The three functions of the base include recognition of proteins tagged with ubiquitin, unfolding the substrate and finally to open the channels in the α-ring. Rpn10 and Rpn13 capture poly-ubiquitinated substrates and function as integral ubiquitin receptors. It should be noted that the 6 ATPases (Rpt1-6) helps to open the gate in the α-ring and allows the substrate to reach the catalytic sites. Further, these ATPases assist in substrate unfolding. The opening of the gate by the proteasome activators PA700 and PA28 activates the 20S proteasomes (P. Chen and Hochstrasser 1996; Tanaka 2009; Finley 2009; Budenholzer et al. 2017).

1.2.2 Recognition of the Substrate and its Processing by the Proteasome

The process of degradation of proteins by the proteasome is quite tangled. It involves the participation of different complexes that regulate this process. As mentioned earlier, the polyubiquitin chains on a substrate serves as a signal for degradation. This tagging of proteins by ubiquitin plays an integral role in the recognition of the protein for degradation and ultimately decides its fate (Finley 2009).

Recognition of Ubiquitin by the Proteasome

Ubiquitin receptors play an important role in the recognition of ubiquitin chains. The two intrinsic ubiquitin receptors are Rpn10 and Rpn13 (subunits of 19S RP). Indeed, the first ubiquitin receptor described was Rpn10 (van Nocker et al. 1996; Deveraux et al. 1994). It is made up of N-terminal von Willebrand factor A (VWA) domain and C-terminal ubiquitin-interacting motifs (UIMs) that facilitates its binding to ubiquitin. On the other hand, the Pru (pleckstrin-like receptor for ubiquitin) domain in the Rpn13 helps in the recognition of the ubiquitin.

The electron cryomicroscopic studies show that Rpn10 is seated near the ATPases and Rpn11 whereas Rpn13 is at the apical location in the RP (Sakata et al. 2012). These proteins have several ubiquitin-binding domains (UBDs) which have different affinities for distinct ubiquitin linkages. Most UBDs have micromolar affinities for tetra ubiquitin chains but lower affinities for a single ubiquitin molecule. Studies in yeast helped in the identification of other UBDs within other intrinsic components of the proteasome namely, Rpt5, and Sem1 (Dss1), however, their contribution is not clear yet (Peth, Uchiki, and Goldberg 2010; Schreiner et al. 2008; Hamazaki et al. 2007).

Other extrinsic receptors such as Rad23, Dsk2, and Ddi1 act as “shuttle factors” as they may capture ubiquitinated substrates remotely from the proteasome and then escort them to the complex (Finley 2009). These receptors use a Ubiquitin-like domain (UBL) and a ubiquitin-associated domain (UBA) domain to bind ubiquitin chains.
Ubiquitin Chain Structure and Targeting of the Ubiquitinated Proteins to the Proteasome

As mentioned above, the K48 linked ubiquitin chains target proteins for degradation. It is known that for a protein to be effectively degraded by the proteasome, it should be tagged with at least four or more K48-linked ubiquitins (Thrower et al. 2000). Biochemical analysis revealed that binding of K48-polyubiquitin chains to the proteasome is facilitated by Rpn10 and Rpn13. Interestingly, the binding of K48-polyubiquitin chains might bind to lower affinity ubiquitin receptors in the absence of Rpn10 and Rpn13. Notably, Rad23 (an Ubl-UBA protein) showed selectivity towards K48-polyubiquitin conjugates and delivered them to the proteasome (Grice and Nathan 2016).

Ubiquitin-independent Protein Degradation

It has been well established that only ubiquitinated proteins are recognized by the 26S proteasome, however, there are some exceptions. An example of a protein that is degraded by the proteasome without prior ubiquitination is Ornithine Decarboxylase (ODC). Its recognition by the 26S proteasome is mediated by a polyamine-induced protein called antizyme (Az). It was also reported that the 20S proteasome degrades ODC by a process regulated by NAD(P)H quinone oxidoreductase 1 (NQO1) (Murakami et al. 1992; Tanaka 2009).

Degradation of p21, a cyclin-dependent kinase inhibitor, garnered an interesting discussion because it’s cell cycle dependent degradation by the 26S proteasome required prior ubiquitination. In contrast, an unregulated, basal degradation of p21 is carried out in a ubiquitin-independent manner (Ciechanover and Stanhill 2014).

1.2.3 Inhibitors of the Proteasome

Proteasome inhibitors have proven themselves as significant tools and have helped in identifying novel substrates of the ubiquitin-proteasome pathway (Kisselev and Goldberg 2001). Owing to the importance of proteasome in various cellular and biological processes, its inhibitors have made an enormous contribution to our understanding of the cellular and biological functions governed by the proteasome (Goldberg 2012). The inhibitors are short peptides which are linked to a pharmacophore. As mentioned earlier, there are six active sites located in the two central β-rings in the 20S core particle and they differ in their specificities. The two ‘caspase-like (C-L)’ located in the β1 subunit preferentially cleave peptide bonds after acidic residues, the two ‘trypsin-like (T-L)’ located in the β2 subunit cleave after basic amino acids and the remaining two ‘chymotrypsin-like (CT-L)’ located in the β5 subunit have a preference for hydrophobic residues (Nussbaum et al. 1998; Dick et al. 1998). The specific interactions of the catalytic subunit with one of the adjacent β subunits result in the formation of substrate binding sites for each catalytic β subunits (Lowe et al. 1995; Kisselev and Goldberg 2001). It was demonstrated in different studies that there was a large reduction in the protein breakdown rate when CT-L site alone was inhibited or mutated (Rock et al. 1994; P. Chen and Hochstrasser 1996; Wolfgang Heinemeyer et al. 1997) while inhibition of the other two sites (C-L and T-L) had only little effect (Wolfgang Heinemeyer et al. 1997; Kisselev et al. 1999; Arendt and Hochstrasser 1997).
The proteasome inhibitors can be classified into several groups such as peptide aldehydes, peptide boronates, peptide vinyl sulfone, and peptide epoxyketone to name a few. **Peptide aldehydes** are the most widely used inhibitors and were the first proteasome inhibitors that were developed (Rock et al. 1994; Vinitsky et al. 1992). They are characterised with fast dissociation rates, rapid oxidation into inactive acids by cells, and a reversible nature. Cysteine and serine proteases are the known targets of these inhibitors. MG132 (Z-Leu-Leu-Leu-al, also known as Cbz-LLL or z-LLL) is a proteasome inhibitor that falls into this category. It has high selectivity and potency against the proteasome. Interestingly, MG132 remains the first choice to study proteasome involvement in a process in cell cultures or tissues owing to its low cost and reversibility of its action (Kisselev and Goldberg 2001). Other peptide aldehyde inhibitors include ALLN, MG115 and PSI, however, they are less potent and selective compared to MG132 (Figure 8).

Compared to peptide aldehydes, **peptide boronates** are much more potent and selective inhibitors of the proteasome (Adams et al. 1998). Bortezomib (Velcade) is an elegant example of peptide boronate inhibitors which has paved its way from basic research to the clinic (Goldberg 2012). Bortezomib has been successfully used in the treatment of cancers such as multiple myeloma (MM) and mantle cell lymphoma (MCL) (Thibaudeau and Smith 2019; Kisselev, van der Linden, and Overkleeft 2012; Schmidt and Finley 2014; Cromm and Crews 2017). It results in a potent inhibition of the CT-L activity with an IC\textsubscript{50} (half maximal inhibitory concentration- a quantitative measure of how much of a particular inhibitory substance is needed to inhibit a given biological process by 50%) of 7nM (S. D. Demo et al. 2007). At high concentrations, it also inhibits T-L and C-L activities, however, again its much less potent when compared to CT-L inhibition.

Peptide vinyl sulfones are irreversible inhibitors of the proteasome (Bogyo et al. 1997). They do not inhibit serine proteases, however, they have been described as cysteine protease inhibitors. The
advantage of using these irreversible inhibitors is that they can be used for proteasome mechanistic studies in different cells and tissues where they act as the sensitive active site probes. An example of vinyl sulfones is MG412. **Epoxyketones**, such as epoxomicin and eponemycin, have been shown to exert biological effects by channelling proteasome inhibition. Epoxomicin displays a preference for CT-L activity while eponemycin reacts with C-L and CT-L sites. These compounds are known to act with both amino and hydroxyl groups of the catalytic N-terminal threonine of the proteasome due to which they are the most selective proteasome inhibitors (de Bettignies and Coux 2010). Carfilzomib is an example of such inhibitors which is in a clinical trial stage (Susan D. Demo et al. 2007).

### 1.3 Crosstalk with other Post Translational Modifications

Post-translational modification (PTM) plays a crucial role in the functional regulation of proteins. Interestingly, different PTMs on multiple residues of one protein may co-ordinate with each other to determine a functional outcome resulting in a PTM crosstalk. Strikingly, Huang et al., had collected 193 PTM cross-talk pairs in 77 human proteins from the literature, tested the location preference and co-evolution at the residue. Their interesting data revealed that cross-talk events occurred preferentially among nearby PTM sites, and the cross-talk pairs had a tendency to co-evolve (Y. Huang et al. 2015). A crosstalk between ubiquitination, phosphorylation, SUMOylation, acetylation has been observed for different proteins. However, these modifications may either work in synergy or may have antagonistic roles. For instance, an impressive number of proteins (498) have been identified to be co-modified by SUMO and ubiquitin. The targets include many enzymatic components of PTM machinery, involved not only in ubiquitination and SUMOylation but also phosphorylation, acetylation, and methylation, suggesting an intricate system of crosstalk between different PTMs (Cuijpers, Willemstein, and Vertegaal 2017). For example, SUMOylation and ubiquitination have been shown to have antagonistic roles pertaining to the regulation of the transcription factor NFκB. NFκB is activated by ubiquitination and proteasome-mediated degradation of IκBα (an inhibitor of NFκB). However, the modification of K21 (which is also the site of ubiquitination) of IκBα by SUMO-1 stabilizes it by blocking its ubiquitination and proteasome-mediated degradation (J. M. Desterro, Rodriguez, and Hay 1998).

Phosphorylation is another post-translational modification which is the primary mechanism for regulating cellular signalling whereas ubiquitination is critically involved in protein degradation. Furthermore, mammalian cells express more than 500 protein kinases, and together with protein phosphatases regulate different cellular processes (J. V. Olsen and Mann 2013). Ubiquitination machinery, on the other hand, consists of ~40 E2 enzymes and more than 600 E3 ligases that facilitate the ubiquitination of proteins in the cell. Therefore, the number of proteins modified by both phosphorylation and ubiquitination in the cell is quite large (Yau and Rape 2016). One such example of cross-regulation between phosphorylation and ubiquitination is that of phosphodegrons (short linear motif that is inert until phosphorylated but generates a binding surface that interacts with a ubiquitin ligase upon phosphorylation), in which one or more phosphorylation sites function in a cis-regulatory manner subsequently promoting ubiquitination of a substrate. Interestingly, PTMs might involve regulation of the machinery of other modification type, for example, phosphorylation activating E3 ubiquitin ligase activity (Holt 2012; Ichimura et al. 2005; Khosravi et al. 1999; Michael
J. Emanuele et al. 2011; Swaney et al. 2013). Taken together, such communications between different modifications have had significant roles in expanding the ubiquitin code and in understanding the regulation of different proteins controlling key cellular functions (Herhaus and Dikic 2015; Swatek and Komander 2016; L. Song and Luo 2019).
Chapter 2   SUMOylation

SUMO (Small Ubiquitin-like MOdifier) is covalently linked to various proteins and is deconjugated by SUMO-specific proteases. Due to the opposing activities of SUMO conjugation and deconjugation, SUMOylation can be highly dynamic. SUMO proteins are ~10kDa in size and are distantly related to ubiquitin (20% identity). SUMO was first identified in mammals where it was found to be covalently linked to the GTPase activating protein RanGAP1 (Rohit Mahajan et al. 1997; Matunis, Coutavas, and Blobel 1996). The long-term fate of the modified protein can be altered by SUMO conjugation, thereby, hugely increasing the complexity of the proteome in eukaryotic cells. SUMO conjugation plays a critical role in most cellular processes such as DNA replication, transcription, cell cycle regulation, chromatin organization, ribosome biogenesis, pre-mRNA splicing, nuclear trafficking, protein degradation, etc. (Droescher, Chaugule, and Pichler 2013; Chymkowitch, Nguéa P, and Enserink 2015; Cubeñas-Potts and Matunis 2013; N. García-Rodríguez, Wong, and Ulrich 2016; Eifler and Vertegaal 2015). Furthermore, defects in the SUMOylation pathway are associated with various diseases such as neurodegenerative diseases, cancer, and heart-failure (Yanfang Yang et al. 2017).

2.1   SUMO Paralogs, Pathway and Enzymology

2.1.1   SUMO Paralogs

In lower eukaryotes (yeast, insects, and nematodes), only one SUMO gene is expressed, however, up to eight versions of SUMO are expressed in plants. In vertebrates four different paralogs namely, SUMO1, SUMO2, SUMO3, and SUMO4 are expressed. In humans, SUMO1-3 are ubiquitously expressed whereas SUMO4 is limited to immune cells, pancreatic islands, and kidneys (Bohren et al. 2004; D. Guo et al. 2004; C.-Y. Wang and She 2008). SUMO2 and SUMO3 are ~96% identical to each other (often referred to as SUMO2/3) whereas SUMO1 shares ~45% sequence identity with SUMO2/3. SUMO4 shares 86% identity with SUMO2 (Figure 9), however, the conjugation of SUMO4 to cellular proteins is not known yet.

SUMO2/3 contains Lys residues near the amino terminus which are used as SUMO acceptor sites. SUMO2/3 are able to form chains efficiently, in vitro and in vivo through Lys11 in the consensus SUMOylation site but also via non-consensus site Lys5 in vitro (Vertegaal 2010). The SUMO2/3 chains were found to accumulate during very high levels of cellular stress such as acute heat shock which suggests their involvement in stress responses (Golebiowski et al. 2009). On the other hand, SUMO1 lacks the SUMO consensus site but can form SUMO chains in the presence of a short E3 ligase fragment in vitro (Pichler et al. 2002a). SUMO1 can SUMOylate SUMO2/3 and rather functions as a chain terminator. Interestingly, it was found that SUMO1 is able to multimerize in
vitro via non-consensus Lys residues at the N-terminal, however, the physiological relevance of these findings remains unclear (Pedrioli et al. 2006).

SUMO2/3 is predominantly localized in the nucleus and PML bodies whereas SUMO1 is localized in the nucleoli, nuclear envelope, and cytoplasmic foci. Further, the dynamics of SUMO1 is slower than SUMO2/3 and the distribution of two paralogs changes rapidly throughout the cell-cycle (Boddy et al. 1996; Matunis, Coutavas, and Blobel 1996; Rohit Mahajan et al. 1997; Ayaydin and Dasso 2004).

![Sequence comparison of SUMO paralogs. From (Citro and Chiocca 2013).](image)

**2.1.2 Regulation of SUMO Isoforms**

The regulation of SUMO isoforms is poorly understood, however, several studies indicate regulation at the transcriptional and post-transcriptional levels. Despite having a ubiquitous expression of SUMO1-3, their level differs across tissues and during development (Loriol et al. 2013; Z. Xu and Au 2005). In cells, free form of SUMO1 is less abundant and it is found mostly conjugated to substrates. In contrast, free forms of SUMO2/3 are found in high levels, however, they get conjugated upon stimuli such as heat shock and arsenic treatment (Lallemand-Breitenbach et al. 2008a; Saitoh and Hinchey 2000; Michael H. Tatham et al. 2008; Weisshaar et al. 2008).

Post translational modifications are important regulators of SUMO proteins. Both acetylation and phosphorylation have been demonstrated to modify SUMO1 (Lallemand-Breitenbach et al. 2008a; Matic et al. 2008). For example, acetylation at Lys37 in SUMO1 and Lys33 in SUMO2 affected specific non-covalent SUMO interactions by neutralising the basic charge of SUMO (Ullmann et al. 2012). Importantly, ubiquitin also modifies SUMO variants such as SUMO3 at Lys 20 and 32 (Lamoliatte et al. 2013) raising the possibility that abundance of SUMO proteins is regulated by ubiquitination of SUMO (Droescher, Chaugule, and Pichler 2013). The identification of SUMO2 chains assembly upon stress serves as the best example of how SUMO pathway is regulated by ubiquitin-dependent degradation. These chains are recognized by SUMO-targeted ubiquitin E3 ligases (STUbLs- discussed later) which contain multiple SUMO interaction motifs (SIMs- discussed later). Indeed, SUMO chains themselves have been shown to be substrates for ubiquitination (Tatham et al. 2008).

**2.1.3 SUMO Pathway**

SUMO is expressed as a precursor protein and maturation is achieved by specific SUMO proteases which expose the C-terminal di-glycine that is critical for conjugation (Hickey, Wilson, and Hochstrasser 2012a). The enzymatic cascade facilitates the conjugation of matured SUMO to the substrates (Figure 10) (Droescher, Chaugule, and Pichler 2013). SUMO is attached to an internal
cysteine of the heterodimeric E1 activating enzyme Aos1/Uba2 in an ATP-dependent step, forming a thioester bond (J. M. P. Desterro, Thomson, and Hay 1997; Erica S. Johnson and Blobel 1997). SUMO is then transferred to the catalytic cysteine of Ubc9, an E2 conjugating enzyme, again resulting in a thioester bond (J. M. P. Desterro, Thomson, and Hay 1997; L. Gong et al. 1997; Erica S. Johnson and Blobel 1997). Ubc9 is able to directly recognize and conjugate SUMO to its substrates through the formation of an isopeptide bond between the C-terminal glycine of SUMO and the ε-amino group of the target lysine (Okuma et al. 1999; R. Mahajan, Gerace, and Melchior 1998). SUMO E3 ligases greatly enhance the efficiency of SUMO conjugation as this reaction is very weak (E. S. Johnson and Gupta 2001; Sachdev et al. 2001; Pichler et al. 2002a). The substrate specificity is ensured by SUMO E3 ligases, however, only a few SUMO E3 ligases are known (Gareau and Lima 2010; Geiss-Friedlander and Melchior 2007; Flotho and Melchior 2013). Substrates can be modified with a single SUMO, with multiple SUMOs or, with SUMO chains. SUMO proteases are able to deconjugate substrates thereby circulating free SUMO back to the conjugation cycle. The consequence of SUMOylation result in changed binding interfaces that can be implicated in diverse protein functions such as intracellular localization, activity, stability and, conformational changes (Droescher, Chaugule, and Pichler 2013).

Figure 10: Schematic representation of the SUMO pathway. From (Droescher, Chaugule, and Pichler 2013)

SUMOylation is a reversible process and the maintenance of balanced SUMO conjugation/deconjugation is critical for the survival of the cell. In knockout experiments in mice, abolition of SUMO conjugation by targeting Ubc9 (Nacerddine et al. 2005) or by preventing deconjugation of
substrates by targeting SUMO proteases SENP1 and SENP2 (J. Cheng et al. 2007; Chiu et al. 2008) resulted in embryonic lethality. Interestingly, SUMO1 knockout mice were viable and all essential functions can be carried out by its paralogs under unchallenged conditions suggesting that the loss of SUMO1 can be partly compensated by SUMO2/3 species (Evdokimov et al. 2008; F.-P. Zhang et al. 2008).

2.1.4 SUMO Enzymology

The enzymatic cascade of SUMO involves sequential action of E1 activating enzyme, E2 conjugating enzyme, and E3 ligase enzyme.

SUMO E1-activating Enzyme and its Regulation

The heterodimer of the proteins Aos1 and Uba2 (also referred to as SAE1 and SAE2 in mammals) constitute the functional SUMO E1 activating enzyme. Based on sequence similarity to the ubiquitin E1 enzyme (Uba1), the SUMO E1 enzyme was originally discovered in S. cerevisiae. According to structural analysis, Uba2 contains three domains: an adenylation domain (adenylation active site), a catalytic domain (Cys 173 responsible for thioester bond formation in the human Uba2) and a Ubl domain with structural similarity to ubiquitin and other Ubl modifiers (Lois and Lima 2005; Pichler et al. 2017).

The SUMO E1 enzyme executes several functions which are essential for SUMO conjugation. It has to provide specificity for SUMO conjugation by selecting SUMO among the ubiquitin-related modifiers such as ubiquitin, Nedd8, SUMO, etc. It then activates the C-terminal glycine of the mature SUMO by adenylation in an ATP-dependent reaction, thereby enabling the attack by the conserved catalytic cysteine on the E1 enzyme to form a highly reactive SUMO-E1 thioester bond. Finally, the SUMO E1 enzyme recognizes the SUMO-specific E2 (Ubc9) and transfers SUMO to the E2 enzyme. Both Aos1 and Uba2 are localized in the nucleus and is consistent with the idea that most protein SUMOylation occurs within the nucleus (Lois and Lima 2005; S. K. Olsen et al. 2010; J. M. Desterro et al. 1999; Pichler et al. 2017).

The highly dynamic SUMO proteome undergoes global changes by the regulation of E1 activity. In general, the formation of a disulfide bridge between catalytic Cysteine residues of both E1 and E2 enzymes is induced by low levels of reactive oxygen species (e.g. H\textsubscript{2}O\textsubscript{2}) and anticancer drugs used for the treatment of acute myeloid leukaemia (AML). This results in transient inactivation of both the enzymes and subsequent deSUMOylation of most cellular substrates (Bossis and Melchior 2006; Bossis et al. 2014).

Furthermore, the E1 activity is controlled by PTMs as well. For example, the interaction of Uba2 subunit and E2 enzyme is impaired by the SUMOylation of the human Uba2 subunit at Lys 236. Moreover, upon heat shock there is a decrease in the Uba2 SUMOylation which correlates with increased global SUMOylation (Truong, Lee, and Chen 2012). Interestingly, following viral infection, the E1 enzyme is targeted for proteasomal degradation by the Cleo adenovirus Gam1 protein (Boggio et al. 2004).
**E2 Conjugating Enzyme**

Ubc9 (Ube2I) is the specific single E2-type conjugating enzyme in the SUMO cycle which is highly conserved from yeast to humans. The function of Ubc9 in the SUMO-conjugating pathway was identified by Johnson and Blobel (Erica S. Johnson and Blobel 1997). It accepts SUMO by interacting with E1 and forms a SUMO-Ubc9 thioester bond. The charged E2 then interacts with the substrate and usually with an E3, and transfers SUMO to the substrate. The E2 enzyme contains binding interfaces for the E1, the substrate, an E3, and also for SUMO (Figure 11) (Pichler et al. 2017).

![Figure 11](image)

Figure 11: Binding interfaces on a surface structure of Ube9 (blue, based on 3UIN). The N-terminus (N-term) of Ube9 binds the E1 (black line) and selected E3s (green line). From (Pichler et al. 2017)

The Ube9 domain consists of four α-helices and one anti-parallel β-sheet formed by four β-strands. Cys93, which forms a thioester linkage with the SUMO C-terminus, is the main catalytic residue within this ~150 aa domain (S. K. Olsen and Lima 2013). Importantly, a knockout of Ube9 is lethal in most eukaryotes (Nacerddine et al. 2005; Hayashi et al. 2002; Nowak and Hammerschmidt 2006; Seufert, Futcher, and Jentsch 1995; al-Khodairy et al. 1995; Schwarz et al. 1998) and Ube9 deficient mice die early in development (Nacerddine et al. 2005).

**E2-substrate interactions**

Ubc9 recognizes a SUMO Consensus Motif (SCM) in the substrate and thus selects the lysine for modification. When in direct contact with the SCM, Ubc9 contributes more to catalysis than to stable substrate binding (Bernier-Villamor et al. 2002; Yunus and Lima 2006). The substrate-E2~SUMO complex stabilization is important in order to achieve an efficient SUMO transfer. It can be achieved by additional E2-substrate binding interfaces, co-factors or E3 ligases. Few of the mechanisms that
stabilize the substrate-E2 interaction and enhance SUMOylation in vitro would be briefly described below (Figure 12) (Pichler et al. 2017).

Figure 12: Mechanisms depicting E2 dependent substrate interactions. From (Pichler et al. 2017)

(A) Constitutive and regulated SCM extensions: The three types of short extensions of SCMs, a hydrophobic cluster SUMOylation motif (HCSM) (Matic et al. 2010), a negatively charged amino-acid-dependent SUMOylation motif (NDSM) (S.-H. Yang et al. 2006), and a phosphorylation-dependent SUMOylation motif (PSDM) (discussed later in the chapter) that mimics negatively charged amino acids upon modification have been proposed to increase the interaction with the E2 enzyme (Hietakangas et al. 2006; Mohideen et al. 2009; Picard et al. 2012) (Figure 12A).

(B) Large interfaces near the SCM: An example supporting this mechanism is the interaction of RanGAP1 and E2. RanGAP1 stably interacts with the E2 and is probably the most efficient in vitro SUMO substrate in the absence of an E3 ligase (Pichler et al. 2002b). Structural studies demonstrated that it contains an additional binding interface close to the SCM that is required for stable E2 binding and efficient modification (Bernier-Villamor et al. 2002). Hence, as a result it is able to stably interact with the E2 (Figure 12B).

(C) A SIM close to the SCM: Proteins interacting non-covalently with SUMO generally contain SUMO-interacting motifs (SIMs). These motifs typically consist of three hydrophobic residues in a sequence of four amino acids which might be flanked by acidic or phosphorylated residues (Hecker
et al. 2006; Kerscher 2007; Miteva et al. 2010). A SIM closer to the SCM could be important for an efficient substrate modification by stabilizing the interaction with the SUMO-charged E2. Few examples of such SUMO substrates include PML, the death-domain-associated protein-6 Daxx, and the ubiquitin-specific protease Usp25 (Knipscheer et al. 2008; Meulmeester et al. 2008; D.-Y. Lin et al. 2006). Moreover, these SIM-mediated interactions with the charged E2 might position the donor SUMO (SUMO\(^3\)) in a closed conformation in a similar manner as bona fide E3 ligases (Figure 12C).

(D) Post translational Ubc9 modifications that regulate substrate interaction: The affinity to substrates with a SIM in close distance to the SCM can be enhanced by the SUMOylation of N-terminal Ubc9 in mammalian cells (for e.g., Sp100) (Knipscheer et al. 2008). Interestingly, the substrate selection is regulated by the acetylation of Ubc9 by exclusions, as it removes the positive charge on Ubc9 required for the interaction with NSDM-containing SUMO substrates (Hsieh et al. 2013) (Figure 12D). Therefore, these mechanisms indeed suggest that SUMO E2 enzyme plays a critical role in selection of substrates.

**Regulation of E2**

Owing to the essential functions of Ubc9 in SUMO conjugation, the global cellular SUMOylation is affected by the regulation of the catalytic activity of Ubc9 which is comparable to the E1. In general, E2 function can be regulated by targeting its expression level or its catalytic activity. For example, the expression levels of cellular Ubc9 was found to be reduced by the viral HPVE6 protein or infection with the bacterium Listeria monocytogenes (Heaton et al. 2011; Ribet et al. 2010). Interestingly, Ubc9 expression was upregulated by microRNAs (miRNAs) expressed in cancer cells (Koh et al. 2016).

The transient disulfide bridge formed between the catalytic cysteines of the E1 and E2 enzymes is another way by which the E2 catalytic activity is directly regulated (Bossis and Melchior 2006; Bossis et al. 2014). Moreover, E2 functions are regulated by PTMs, for example, the Ubc9-SUMO thioester formation appears to be promoted by the phosphorylation at Ser71 or Thr35 in E2 (C. H. Lin, Liu, and Lee 2016; Su et al. 2012). Additionally, some members of SUMO-like proteins with important functions in DNA repair and the maintenance of genome stability constitute the Ubc9 regulatory proteins. Examples of such proteins include the RENi protein family (Rad60 in fission yeast, Esc2 in baker’s yeast and NIP45 in mammals) and, the ubiquitin protease USP1/UAF1 (Novatchkova et al. 2005; K. Yang et al. 2011).

**SUMO E3 Ligases**

The transfer of SUMO from the charged E2 enzyme (Ubc9-SUMO) to the substrate is catalyzed by the E3 ligases. In the SUMO pathway, E3 ligases interact with Ubc9-SUMO and the substrate which brings them in close proximity and thereby, enhance the substrate SUMOylation. Notably, indirect effects like regulatory co-factors or inhibition of SUMO proteases might contribute to enhancement of substrate SUMOylation in cells. Therefore, such an enhancement of SUMOylation does not
necessarily correspond to E3 ligase function. Despite the similarity in the mechanism for conjugation of SUMO and ubiquitin, only handful of SUMO E3 ligases have been identified. Based on comprehensive biochemical and structural analysis which allow insights into their mode of SUMO catalysis, there are three classes of E3 ligases namely, the SP-RING (Siz/Pias) family, RanBP2 and, the ZNF451 family (Figure 13) (Kerscher 2007; Hay 2005; Pichler et al. 2017).

**Figure 13 : Bona fide classes of E3 ubiquitin ligases. From (Pichler et al. 2017)**

**SP-RING family**

The Siz1 and Siz2 in S. cerevisiae together with their mammalian homologs, the proteins inhibitor of STAT (PIAS) family were the first SUMO E3 ligases to be identified (E. S. Johnson and Gupta
A zinc finger domain which is structurally related to that of RING and U-box domain containing ubiquitin E3 ligases is shared by all PIAS and Siz proteins. Hence, this group of SUMO E3 ligases was named Siz/PIAS (SP)-RING family. In addition, the methyl methanesulphonate-sensitivity protein Mms21/Nse2, a subunit of the Smc5/6 complex, contains a SP-RING but is unrelated to Siz/Pias proteins (Potts and Yu 2005; X. Zhao and Blobel 2005; Andrews et al. 2005). Till date, it is the only SUMO E3 ligase family which is evolutionary conserved from yeast to human. In S. cerevisiae, the SP-RING family members are represented by Siz1, Siz2, and Nse2 whereas the vertebrate homologs are represented by Pias1, its splice variant Pias2, Pias3, Pias4, and Mms21.

In addition to the central SP-RING, the PIAS family also comprises the N-terminal SAP (scaffold attachment factor-A/B, acinus, and PIAS) domain, a PINIT (Pro-Ile-Asn-Ile-Thr) motif, a unique C-terminal domain (SP-CTD), a SIM, and a variable serine/threonine-rich C-terminal region (S/T) (Droescher, Chaugule, and Pichler 2013; Yunus and Lima 2009; Rytinki et al. 2009). The SAP domain, the PINIT domain, and also the C-terminus participate in protein interactions, and in the regulation of intracellular localization and substrate modification in vivo, however, these domains are dispensable for core E3 functions in vitro. In general, SP-RING proteins play an important role in cellular processes such as DNA repair (X. Zhao and Blobel 2005; C.-S. Wu and Zou 2016; S. Liu et al. 2013; Galanty et al. 2009; Morris et al. 2009), cell cycle (E. S. Johnson and Gupta 2001; C. Zhang et al. 2010; Munarriz et al. 2004), apoptosis (C. Zhang et al. 2010), transcriptional regulation (Sharrocks 2006; Palvimo 2007), oxidative stress response (Leitao, Jones, and Brosens 2011).

RanBP2/Nup358

Ran-binding protein 2 (RanBP2) constitutes the second class of SUMO E3 ligases. It is a large 358-kDa core component of the cytoplasmic filaments of nuclear pore complexes (NPCs) which has key functions in nuclear transport and mitosis. It comprises of N-terminal leucine-rich domain that anchors it to the NPCs, four Ran GTPase binding sites, eight tandem zinc fingers, two internal repeats displaying the SUMO E3 ligase domain IR1-M-IR2, multiple FG repeats, the binding sites for transport receptors and a cyclophilin-like domain (J. Wu et al. 1995; Yokoyama et al. 1995). The E3 ligase domain of RanBP2 is absent in worm and fly but is conserved in human, mouse, bovine, chicken and frog (J. W. C. Chen, Barker, and Wakefield 2015). The key functions of the E3 ligase activity of RanBP2 were found in mitosis. RanBP2 is largely soluble during mitosis and a fraction was found in a complex with SUMOylated RanGAP1 at kinetochores and the mitotic spindle (Salina et al. 2003; Joseph et al. 2004; Swaminathan et al. 2004). Reduced RanBP2 expression levels resulted in severe aneuploidy caused by the formation of anaphase bridges and chromosomal segregation defects while the knockouts of RanBP2 in mice were embryonic lethal (Aslanukov et al. 2006).

The ZNF451 Family

Recently discovered ZNF451 family has a unique specificity for the SUMO2/3 paralogs and is vertebrate-specific. The members of this family depend on a tandem SIM and its inter-SIM PxRP motif to discharge SUMO-Ubc9 (Cappadocia, Pichler, and Lima 2015; Eisenhardt et al. 2015). This
family is mostly uncharacterized. The human ZNF451 gene locus encodes three isoforms (ZNF451-1, ZNF451-2, and ZNF451-3) which share an identical N-terminal tandem-SIM region up to amino acid 63. KIAA1586 represents an additional member of this family and is situated adjacent to the ZNF451 gene locus. It shares a nearly identical N-terminal catalytic tandem-SIM domain with only one aa substitution. ZNF451-1, ZNF451-3, KIAA 1586 and the minimal catalytic tandem-SIM region can extend SUMO chains \textit{in vitro} (Eisenhardt et al. 2015) and interestingly, in various cellular screens, all ZNF451 family members were found to be highly modified SUMO2 substrates (Hendriks et al. 2014; Tammsalu et al. 2014).

Partially located in the PML bodies, ZNF451-1 functions as a transcriptional regulator. ZNF451-1 has no intrinsic transcriptional activity, however, it was found to interact with the androgen receptor and Smad3/4 to co-regulate their respective target genes (Karvonen et al. 2008; Feng et al. 2014). Furthermore, ZNF451-1 in cooperation with RNF4 regulates endogenous PML levels, which limits the cellular PML body numbers. PML was the first \textit{in-vitro} substrate identified for ZNF451-1’s SUMO E3 ligase activity. Other key functions for this family members includes SUMO2/3 chain assembly, in stress-induced SUMOylation such as proteasome inhibition and in the DNA damage response (Eisenhardt et al. 2015).

\textit{Substrate and SUMO isoform specificity}

Currently, around 6000 SUMO substrates have been identified in human cells with 10 human E3 ligases (Hendriks et al. 2017). Therefore, the execution of substrate specificity by the SUMO E3 ligases remains one of the most obscure SUMO topics. The global SUMO proteome is highly dynamic and undergoes constant changes during the cell cycle or upon different stress stimuli. This results in stimulus-specific group SUMOylation and deSUMOylation. In budding yeast, E3 enzymes Siz1 and Siz2, contribute to the global levels of SUMO conjugates and share overlapping substrate specificity (Reindle et al. 2006). This overlap is mainly due to the local concentrations of the E3 enzymes in the cell and their ability to stimulate SUMO transfer in reactions wherein specificity is primarily dictated by substrate interactions with the E2 rather than the E3. On the other hand, some E3 ligases do exhibit specificity, and they are required for modification of substrates by SUMO, including cases in which modification occurs on non-consensus site Lys acceptors (Gareau and Lima 2010).

For example, both types of E3 activity can be illustrated by SUMOylation of proliferating cell nuclear antigen (PCNA), the processivity factor for eukaryotic DNA polymerases, in budding yeast. PCNA is modified on two Lys residues, Lys127 and Lys164 both \textit{in vitro} and \textit{in vivo}. Siz1 stimulates the SUMO modification of Lys127 within the canonical SUMO consensus motif of PCNA, however, in the absence of Siz1, it can also be modified by Siz2. In contrast, Lys164 undergoes modification only in the presence of Siz1. The specificity in this case is dependent on the PINIT domain of Siz1 which is assumed to interact directly with a loop on PCNA to position Lys164 near the E2-SUMO thioester (Gareau and Lima 2010). This exemplifies specific E3-substrate pairing, however, the choice of which SUMO isoform to use during modification adds another complexity to the E3-imparted specificity. For example, some E3 enzymes have been shown to modify particular substrates with a certain SUMO isoform-such as PIASγ conjugates SUMO1, not SUMO2/3, and RanBP2 stabilizes
RanGAP1 at the nuclear pore complex only after modification by SUMO1 (Matunis, Coutavas, and Blobel 1996; Rohit Mahajan et al. 1997).

2.1.5 SUMO Proteases

DeSUMOylation involves the removal of SUMO residues by a distinct set of catalysts called sentrin-specific proteases (SEPNs). Initially described in *S. cerevisiae* as ubiquitin-like-specific protease 1 and 2 (ULP1 and ULP2) (S.-J. Li and Hochstrasser 1999; Reindle et al. 2006), the human genome encodes six distinct SENP family members (SENP1, 2, 3, 5, 6, and 7) (Shin et al. 2012). The striking feature of SENP1, SENP2, SENP3, and SENP5 is that they act as prerequisite processing enzymes for the maturation of SUMO. They cleave the C-terminal portion of the small protein, thereby exposing a diglycine motif and rendering it for ligation via isopeptide bond formation. Recently, two new SUMO protease classes have been discovered namely, deSUMOylating isopeptidases (DESI) 1 and 2 (Shin et al. 2012), and ubiquitin-specific protease-like 1 (USPL1) (Schulz et al. 2012). The above listed enzymes are cysteine isopeptidases with SENPs (clan CE/family C48) harboring a conserved core and a catalytic triad containing histidine, aspartate, and cysteine residues. DESIs (clan CP/family C97), in contrast, bear a catalytic cysteine/histidine dyad among other structural differences. Interestingly, USPL1 (clan CA/family C98) is distantly related to DUBs and shares no similarity with both SENPs and DESIs (Schulz et al. 2012).

*In vitro* studies have demonstrated that SUMO2 and SUMO1 precursors are processed proficiently by the catalytic domains of SENP2 and SENP1, respectively. SUMO3, on the other hand, is processed by SENP5 (Shen et al. 2006; Bacco et al. 2006; Z. Xu and Au 2005). The structural characterization of the catalytic domains of SENP1 and SENP2 have revealed that residues in the precursor tails of different paralogs influence their processing. *In vivo*, subcellular localization of the SUMO proteases as well as their interaction with SUMOylated substrates regulate the isopeptidase activity or deconjugation of SUMOylated proteins. Table I displays the subcellular localization for most of the human SENPs along with their SUMO paralog preference, however, the mechanisms regulating their subcellular localization are mostly unknown (Hickey, Wilson, and Hochstrasser 2012a).

Furthermore, SUMO proteases can be regulated by mechanisms that include transcription, phosphorylation, proteasomal degradation, stimuli such as oxidative stress, oxygen deprivation/hypoxia, and upon heat shock (Hickey, Wilson, and Hochstrasser 2012a). For example, mammalian SENP3 is phosphorylated and constitutively degraded by the E3 ubiquitin ligase C-terminus of HSP70-interaction protein (CHIP). Under genotoxic stress, SENP2 gets activated which leads to its own transcriptional down-regulation in a negative feedback loop. Interestingly, enzymes of the SUMO conjugation machinery can be reversibly inactivated by low levels of reactive oxygen species (ROS). At low levels of ROS, a reversible inhibition of SENP1 and Ulp1 was observed with subsequent irreversible inactivation at higher levels, suggesting the proteases could possibly function
as redox sensors in cells. In contrast, heat shock and induced ischemia contribute to global increase in SUMO2/3 conjugation (S. Yan et al. 2010).

Table I: Properties of human SUMO proteases. From (Hickey, Wilson, and Hochstrasser 2012)

<table>
<thead>
<tr>
<th>Name</th>
<th>Subcellular Localization</th>
<th>SUMO Prefer.</th>
<th>Precursor Process.</th>
<th>Deconjugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SENP1</td>
<td>Nuclear pore and nuclear foci</td>
<td>SUMO1 and SUMO2/3</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>SENP2</td>
<td>Nuclear pore and nuclear foci, cytoplasm</td>
<td>prefers SUMO2/3 more than SUMO1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>SENP3</td>
<td>nucleolus</td>
<td>SUMO2/3</td>
<td>unknown</td>
<td>Yes</td>
</tr>
<tr>
<td>SENP5</td>
<td>nucleus and mitochondria</td>
<td>SUMO2/3</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>SENP6</td>
<td>nucleoplasm</td>
<td>SUMO2/3</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>SENP7</td>
<td>nucleoplasm</td>
<td>SUMO2/3</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>DESI1</td>
<td>nucleus and cytoplasm</td>
<td>SUMO1 and SUMO2/3</td>
<td>Weak</td>
<td>Yes</td>
</tr>
<tr>
<td>USPL1</td>
<td>cajal bodies</td>
<td>prefers SUMO2/3 more than SUMO1</td>
<td>Weak</td>
<td>Yes</td>
</tr>
</tbody>
</table>

SUMO proteases are critically involved in cellular processes such as DNA repair, transcriptional regulation, and ribosome biogenesis. They are known to play a role in oncogenesis and tumour progression. For example, overexpression of SENP1 has been correlated with aggressiveness of prostate cancer and metastatic potential. It activates other oncogenic pathways such as c-Jun and androgen-receptor-mediated transcription. Similar to SENP1, accumulation of SENP3 has been found in several human cancers such as colon carcinomas (Hickey, Wilson, and Hochstrasser 2012b). Altogether, these data suggest a potential significance for SENPs as diagnostic markers and also make this enzyme class an attractive drug target in human tumours.

2.2 SUMO Consensus Sequence

SUMO substrates share a common Ψ-K-x-(D/E) motif where Ψ is a hydrophobic residue with high preference for I or V, K is the substrate lysine, x is any amino acid and D or E is an acidic residue (Vertegaal 2010; Kerscher 2007; Hay 2005; Hannoun et al. 2010; Pichler et al. 2017). This motif is designated SUMO consensus motif (SCM). At physiological conditions, high resolution mass spectrometry data have revealed (Hendriks and Vertegaal 2016) that at least half of the SUMO substrates are modified at the minimal KxE motif, however, upon stress, more lysines at non-SCM
sites can be modified. The direct interaction of SCMs with the catalytic cleft of the E2 enzyme contributes to catalysis and to lysine selection (Bernier-Villamor et al. 2002; Yunus and Lima 2006). An efficient SUMO transfer is achieved when this interaction is stabilized by either additional binding interfaces or co-factors, or with the help of an E3 ligase. An extended and regulated variations of SCM motif include a HCSM, NDSM, and PDSM. These SCM motif variants lead to an increase in E2 affinity and enhanced modification in vitro. SUMO acceptor sites are displayed in Table II (Flotho and Melchior 2013). Notably, these motifs are also found in many non-SUMOylated proteins suggesting that a SUMO substrate cannot be defined by their presence. The SUMOylation of SCM depends on the structural context and requires extended or unstructured and exposed surface regions (Pichler et al. 2005).

Table II : SUMO acceptor sites. From (Flotho and Melchior, 2013)

<table>
<thead>
<tr>
<th>Motif</th>
<th>Consensus sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consensus motif</td>
<td>( \Psi )-K-x-(D/E)</td>
</tr>
<tr>
<td>Inverted SUMOylation Consensus motif (ISCM)</td>
<td>(D/E)-x-K-( \Psi )</td>
</tr>
<tr>
<td>Hydrophobic Consensus SUMOylation motif (HCSM)</td>
<td>( \Psi )-( \Psi )-K-x-(D/E)</td>
</tr>
<tr>
<td>Phosphorylation-dependent SUMOylation motif (PDSM)</td>
<td>( \Psi )-K-x-(D/E)-x-x-(pS)P</td>
</tr>
<tr>
<td>Negatively charged amino acid-dependent SUMOylation motif (NDSM)</td>
<td>V-K-x-(D/E)-x-x-E-E-E-E-E</td>
</tr>
</tbody>
</table>

2.3 Non-covalent interactions with SUMO

While interactions between the E2 and the substrate are mediated by SUMO consensus motifs, SUMO Interacting Motifs (SIMs also referred to as SUMO binding motifs-SBM or domains-SBD) mediate non-covalent interactions between SUMO and SIM-containing proteins (Miteva et al. 2010; Hecker et al. 2006; Praefcke, Hofmann, and Dohmen 2012). SIMs were identified through a yeast two-hybrid screen and biophysical studies and are characterized by a short stretch of hydrophobic amino acids, Val/Ile-X-Val/Ile-Val/Ile (V/I-X-V/I-V/I), which are flanked by acidic residues allowing SUMO to bind to SIMs (Figure 14) (Miteva et al. 2010). SIM adopts a parallel or anti-parallel \( \beta \)-strand conformation that extends the SUMO \( \beta \)-sheet allowing the hydrophobic side chains of the SIM to interact with a hydrophobic pocket on the SUMO surface. SIMs are found in a wide range of proteins that includes SUMO enzymes (PIASX and RanBP2/Nup358) (Hannoun et al. 2010; Pichler et al. 2002a; J. Song et al. 2004), SUMO-activating enzyme subunit Uba2/Sae2, SUMO substrates (PML, transcription factor-DAXX) (X. Cheng and Kao 2013; Santiago et al. 2009), SUMO-binding proteins (transcriptional co-repressor-ZMYM2) (Aguilar-Martinez et al. 2015), and SUMO-targeted ubiquitin ligases (RNF4, Arkadia/RNF111) (Michael H. Tatham et al. 2008; Erker et al. 2013). In the following paragraphs, I would describe features of SIMs facilitating the binding to SUMO and how SIMs regulate SUMO conjugation machinery, SUMO substrates, and STUbLs.

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Further, I would also mention the softwares which facilitate the prediction of SUMO sites and binding motifs.

### 2.3.1 Types and Features of SIMs influencing their binding to SUMO

**Types of SIMs**

Based on the conserved residues, most experimentally proven SIMs are classified into three major SIM types (Figure 14)

(i) **SIMa**: It is characterized by four consecutive hydrophobic residues followed by a mixed cluster of Ser/Asp/Glu residues, however, there might be variability at the third hydrophobic position. This particular position is less conserved than the other hydrophobic positions and can accommodate non-hydrophobic residues as well.

(ii) **SIMr**: It resembles the SIMa but has a reversed orientation i.e. acidic cluster is followed by four hydrophobic positions. Here, the second hydrophobic position (corresponding to the third position in SIMa) is the most variable one and could occasionally accommodate non-hydrophobic residues.

(iii) **SIMb**: SIMb is shorter than SIMa but is easier to recognize and is better conserved. Mostly, these SIM types stick to the consensus sequence V-I-D-L-T, with some variability in the first two hydrophobic positions. The third position has a strong preference for Asp. Similar to SIMa, several SIMb motifs are followed by a serine/acidic region, however, this stretch is not crucial for its function as seen in RNF4 family that contains non-acidic SIMb motifs (Miteva et al. 2010).

![Figure 14: Residue conservation of the three SIM types. Residues are represented by different colors. Black: charged residues; green: polar residues; blue: hydrophobic. From (Miteva et al. 2010)](image-url)
Features of SIMs

SIMs are hydrophobic: The interactions between individual hydrophobic and aromatic amino acids of SUMO-1 and the SIM-containing PIASX peptide were revealed when they were in complex with each other through structural studies done by Song et al. (J. Song et al. 2005). These residues are arranged in a SIM-binding groove of the SUMO molecule for both SUMO1 and SUMO2 (Figure 15). SIMs assume an intramolecular β-sheet structure with the β2 strand of SUMO and hydrophobic residues in SUMO1 (Phe36, Val38, and Leu47) have been found to interact with SIMs. Interestingly, a single amino acid change in the hydrophobic core of a SIM is enough to greatly reduce its interaction with SUMO (Baba et al. 2005; Hecker et al. 2006).

Cluster of charges: Many but not all SIM-containing proteins have a cluster of acidic amino-acid residues i.e. aspartic and glutamic acid adjacent to the hydrophobic core (Figure 16). The electrostatic interactions promoted by these residues play an important role in the affinities, orientation, and functionality of SIM-SUMO associations (Hecker et al. 2006; J. Song et al. 2005). Interestingly, it has been proposed that phosphorylation might be one way to introduce negative charges into SIMs as Ser and Thr residues can be found adjacent to many hydrophobic SIM domains. These residues could then interact with lysine residues in SUMO, for eg. Lys39, which is present in all three mammalian SUMO isoforms (Hecker et al. 2006).

A choice of orientation: There is a variability in the hydrophobic core composition (Figure 17A) as well as in the placement of charged amino acid (Figure 15). Intriguingly, owing to this variability, SIMs can bind SUMO in either a parallel or an anti-parallel orientation with respect to the β2 strand of SUMO (Figure 17B) (Kerscher 2007). For example, in TDG and RanBP2, the binding of SUMO to SIMs occurs in an anti-parallel orientation whereas in PIAS protein, it occurs in parallel orientation. Therefore, the binding orientation can be governed by the hydrophobic core and its charge distribution (Baba et al. 2005; J. Song et al. 2005; Kerscher 2007).
Preference for SUMO Paralogs: In SUMO paralogs, critical hydrophobic and basic residues involved in SIM binding are conserved, however, they might differ in the placement of the hydrophobic groove. The crystallographic analysis in SUMO1, SUMO2, and yeast Smt3 revealed that basic residues surrounding the hydrophobic groove help to accept the SIM domain and the negative charges associated with them. Interestingly, the hydrophobic grooves in these isoforms occupy slightly different positions with respect to the basic residues. Therefore, this suggests that the arrangement of hydrophobic and acidic residues in SIMs might govern their ability to bind specific SUMO paralogs. Interestingly, SIMs in a substrate might also dictate its specificity towards a SUMO paralog. For example, the SIM in ubiquitin-specific protease (USP25) shows a preference for SUMO2/3 (Chupreta et al. 2005).

![Diagram](image)

**Figure 16:** Schematic representation of possible combination of hydrophobic (h) core of the SIM (often valines or isoleucines) and X is any amino acid. Acidic residues or phosphorylated serines or threonines are represented by blue spheres. From (Kerscher et al. 2007)

Preference for SUMO Paralogs: In SUMO paralogs, critical hydrophobic and basic residues involved in SIM binding are conserved, however, they might differ in the placement of the hydrophobic groove. The crystallographic analysis in SUMO1, SUMO2, and yeast Smt3 revealed that basic residues surrounding the hydrophobic groove help to accept the SIM domain and the negative charges associated with them. Interestingly, the hydrophobic grooves in these isoforms occupy slightly different positions with respect to the basic residues. Therefore, this suggests that the arrangement of hydrophobic and acidic residues in SIMs might govern their ability to bind specific SUMO paralogs. Interestingly, SIMs in a substrate might also dictate its specificity towards a SUMO paralog. For example, the SIM in ubiquitin-specific protease (USP25) shows a preference for SUMO2/3 (Chupreta et al. 2005).

![Diagram](image)

**Figure 17:** A. Hydrophobic core amino acids of bona fide SIM domains in thymine DNA glycosylase (TDG), DAXX, and promyelocytic leukaemia (PML). B. Depiction of SIM insertion in two possible orientation (parallel-middle or anti-parallel) depending on the arrangement of hydrophobic amino acids and negative charges juxtaposed to the hydrophobic groove of SUMO. Red plus sign indicates charged lysine (Lys78) present in SUMO1 which might play an important role in orienting phosphorylated SIMs. From (Kerscher et al. 2007)

2.3.2 Regulation of SUMO substrates and SUMO-binding proteins by SIMs

SUMO modification of a number of substrates can be mediated by SIMs resulting in changes in their activity and/or localization. For example, the direct binding of PML to SUMO and Ubc9 is well known. PML contains SIMs and SUMOylation of PML is necessary for the formation of PML-nuclear bodies (NBs) which are proteinaceous structures found predominantly in the nucleus, tightly bound to the nuclear matrix. Interestingly, a PML mutant that cannot be modified by SUMO fails to
recruit PML-NBs components such as SP100, a protein involved in transcriptional regulation, and DAXX, a transcriptional repressor. Furthermore, overexpression of a SUMO protease, SuPr-1, induces both the reorganization of PML-NBs into fewer and larger aggregates and the redistribution of several PML-NB-associated proteins including DAXX (Best et al. 2002; Bernardi and Pandolfi 2007; X. Cheng and Kao 2013; Lallemand-Breitenbach and de Thé 2010).

Interestingly, Barbara Mojsa, a former PhD student in the group, demonstrated that Trim17 (described in detail in the next chapter) harbours two conserved motifs, SIM1 (117-ICVV-120) and SIM2 (390-VVQL-393). Indeed, Trim17 interacts with its partner, NFATc3 in a SUMO-dependent manner as mutation of SIM2 or both SIMs strongly decreased the interaction between Trim17 and NFATc3 (Mojsa et al. 2015a).

Phospho-SIMs, a subclass of SIMs containing Ser residues that are also sites of phosphorylation, have been characterized in proteins such as PML. In PIAS proteins, phospho- SIMs help in their regulation. In such cases, Casein Kinase2 (CK2), a ubiquitously expressed constitutively active kinase phosphorylates the Ser residues C-terminal to the SIM. CK2 enhances the non-covalent interactions with SUMO through contacts with multiple lysine residues on the SUMO surface by facilitating the phosphorylation. Such Ser residues have been found in other proteins such as UBA2 subunit of SUMO E1, however, it remains unclear if phosphorylation is required to enhance interactions with a SUMO protein (Stehmeier and Muller 2009).

2.3.3 SIMs in the SUMO conjugation machinery

SUMO conjugation enzymes such as SUMO E1 and several SUMO E3 ligases contain SIMs. The C-terminal extension of the SUMO E1 UBA2 subunit contain two distinct SIMs. Despite the similarities of E1 SIM-SUMO interactions with other SUMO-SIM complexes, the functional significance of the SIMs in E1 remain unclear. Indeed, the entire C-terminal extension is dispensable for the human E1 activity in vitro and for the function of the E1 in S. cerevisiae (Gareau and Lima 2010).

The SUMO E3 ligase, RanBP2, contains two SIMs in its E3 ligase domain namely IR1 and IR2. RanBP2, in a complex that contains SUMO1-modified RanGTPase-activating protein1 (RanGAP1-its substrate) and UBC9 (E2), mediates non-covalent interactions with SUMO1 via IR1 SIM. Importantly, there was a decrease in the E3 ligase activity of RanBP2 when its SIM was deleted which suggested that the non-covalent interactions were important not only for interactions with RanGAP1 but also with a charged E2-SUMO thioester complex during the conjugation of other substrates (Tang et al. 2008; Flotho and Werner 2012). Therefore, SIMs in the enzymes involved in the SUMO pathway play an important role in the regulation of their activity.

2.3.4 SUMO-Targeted E3 Ubiquitin Ligases (STUbLs)

STUbL’s also known as ubiquitin ligases (E3) for sumoylated proteins (ULS, E3-S) are a class of RING-type ubiquitin ligases that recognize SUMO modified proteins via their SIMs and ubiquitinate them (Figure 18) (Hickey, Wilson, and Hochstrasser 2012a). Through STUbLs, sumoylation serves
as a recognition signal for a class of ubiquitin ligases. STUbLs are a combination of two features: a RING domain that mediates interaction with an E2 ubiquitin-conjugating enzyme and which identifies them as ubiquitin ligases, and SIMs that characterize them as interactors of SUMO (Sriramachandran and Dohmen 2014). Most of the STUbLs studied so far contain multiple SIMs, which mediate cooperative binding to multiple SUMO units, hence, providing a preference for substrates with SUMO chains (Uzunova et al. 2007; Michael H. Tatham et al. 2008; Erker et al. 2013; Jansen and Vertegaal 2020). Few of the STUbLs would be described below.

Slx5-Slx8 heterodimer in S. cerevisiae

Uls2, a DNA binding heterodimer formed by the two RING finger proteins Slx5 and Slx8, is one of the STUbLs identified in S. cerevisiae (Uzonova et al., 2007; Hannich et al., 2005; Xie et al., 2007). Slx5 contains multiple SIMs of the α- and β-type whereas only a single SIMα is present in Slx8 (Uzunova et al. 2007; Tatham et al. 2008; Erker et al. 2013). Uls2 cooperates with E2 enzyme Ubc4 or its paralog Ubc5 to target poly-sumoylated proteins for degradation by the proteasome and it appears to be one important function of Uls2 (Uzunova et al. 2007). Interestingly, Slx5 and Slx8 are linked to DNA damage repair and genome stability. It was observed that mutations in the SLX5 or SLX8 genes were synthetically lethal with various mutations affecting SUMO conjugation linking this enzyme to the SUMO system (C.-H. Cheng et al. 2006a).

Rnf4 in humans

The most extensively studied STUbL is the human Rnf4 protein (Weisshaar et al. 2008; Michael H. Tatham et al. 2008; Lallemand-Breitenbach et al. 2008a) also referred to as small nuclear RING finger protein (SNURF) (Moilanen et al. 1998). Rnf4 contains at least three clear SIMs (Fig. 19) (Sriramachandran and Dohmen 2014). SIMs in Rnf4 mediate similar binding to SUMO1 and
SUMO2, however, they seem to have a preference for polymeric SUMO chains (Michael H. Tatham et al. 2008). The first physiological substrate of Rnf4 identified was PML. After the treatment with arsenic trioxide (ATO), PML protein as well as its oncogenic variant PML-RARα (a fusion protein with retinoic acid receptor α expressed as a result of a chromosomal translocation in patients with acute promyelocytic leukaemia) were shown to be degraded in a SUMO-dependent manner by the proteasome. It was revealed that Rnf4 is a ubiquitin-ligase mediating ATO-induced proteolytic targeting of PML (Michael H. Tatham et al. 2008; Lallemand-Breitenbach et al. 2008b).

In vitro, Rnf4 promoted the ubiquitination of sumoylated PML and disrupted PML nuclear bodies in cells treated with ATO (Weisshaar et al., 2008; Tatham et al., 2008; Lallemand-Breitenbach et al., 2008). ATO binds directly to PML, resulting in PML oligomerization and an increased affinity to Ubc9. The polySUMOylated PML is then targeted for degradation by the proteasome via Rnf4-mediated ubiquitination. The lysine residues of both conjugated SUMO and PML were sites for ubiquitination of SUMOylated PML by Rnf4. Interestingly, Rnf4 localized in PML-NBs in a SIM- and SUMO2/3-dependent manner (Michael H. Tatham et al. 2008; Häkli et al. 2005; Geoffroy et al. 2010). Apart from PML, several other substrates of Rnf4 have been identified which include kinetochore protein CENP-1 (Mukhopadhyay et al., 2010), hypoxia-inducible factors (HIFs: Hif1α and Hif2α) (Chua et al. 2010), and transcription factor Pea3 (B. Guo and Sharrocks 2009).

Arkadia/RNF111 in humans

The RING finger protein Arkadia (also known as RNF111) was identified using a bioinformatic approach (Figure 19). RNF111 contains at least 3 closely spaced functional SIMs of type-b class.
and mediated strong binding of artificial linear SUMO1 or SUMO2 chains suggesting that the function of RNF111 involves the recognition of poly-SUMO signals (Sun and Hunter 2012). RNF111 is a ubiquitin ligase that targets negative regulators such as SMAD7, c-Ski or SnoN for degradation and promotes TGF-β (transforming growth factor β) signaling (Inoue and Imamura 2008). Interestingly, many key players in the TGF-β signaling pathway such as TGF-β receptor, Smad3, Smad4, and Axin were reportedly modified by SUMOylation (Kang et al. 2008; M. J. Kim, Chia, and Costantini 2008; P. S. W. Lee et al. 2003; Long et al. 2004). Therefore, it is a possibility that RNF111 behaves as a STUbL in the TGF-β pathway.

Importantly, in another study, PML was identified as one of the in vivo substrates of RNF111. Depletion of RNF111 led to an accumulation of poly-SUMOylated PML upon ATO treatment. This effect was synergistic with Rnf4 depletion. Therefore, it was suggested that both Rnf4 and RNF111, which form homo-dimers but not hetero-dimers with each other, contribute to ATO-induced and SUMO-dependent degradation of PML (Erker et al. 2013).

_Degringolade in Drosophila Melanogaster_

A protein related to Rnf4, Degringolade (Dgrn) (Figure 19), has been identified in _Drosophila_ as a negative regulator of the developmental transcription factor _Hairy_ (plays an important role during embryonic segmentation and neurogenesis) and its co-repressor Groucho (Fischer and Gessler 2007; Abed et al. 2011; Barry et al. 2011). The embryonic lethality of hairy mutants is supressed by mutations in Dgrn. Dgrn binds to a basic region in Hairy via its RING domain and simultaneously, it also binds to SUMOylated Groucho through its SIMs. Interestingly, the ubiquitination of Groucho was not detected (Abed et al. 2011). Therefore, it is an example of a mechanism in which the SUMO-SIM interaction cooperates with another binding site to provide specific recognition of a substrate by a STUbL.

### 2.4 Crosstalk with other Post-translational Modifications

The overlap of tandem mass spectrometry-identified SUMO sites with other PTMs, such as ubiquitination, acetylation and methylation has been described in several studies (Abed et al. 2011). Indeed, Hendriks et al. found that ~29% of all 7,710 SUMOylation sites overlapped with one of the three PTMs and ~24% overlapped with ubiquitination. Given that the human proteome corresponds to ~650,000 of Lys residues (Hendriks et al. 2014), ~7,700 SUMOylation sites and ~33,000

![Figure 20](image_url)  
Figure 20 : Lys post-translational modifications (PTMs) such as ubiquitination (Ub), acetylation and methylation can compete with SUMOylation (S). From (Hendriks and Vertegaal 2016).
ubiquitination sites, this overlap is approximately 5-fold higher than expected randomly and is highly significant. These overlaps also exist between SUMOylation and acetylation and methylation, and this might indicate a PTM competition on the same Lys residues. Interestingly, the same lysine residue might be targeted by multiple PTMs, therefore, Lys mutants for specific SUMO targets should be generated carefully (Figure 20) (Hendriks and Vertegaal 2016).

Strikingly, the existence of phosphorylation-dependent SUMOylation (Figure 21A) (Hietakangas et al. 2006) and acetylation-dependent SUMOylation (Hendriks et al. 2014) indicate a collaborative crosstalk between SUMO and other PTMs. Additionally, the enzymatic machinery associated with other PTMs such as ubiquitin ligases, ubiquitin proteases, methyltransferases, demethylases, acetyltransferases, deacetylases, kinases and phosphatases are modified by SUMO which regulates their function (Figure 21B). Examples include SUMOylation of MEK (a serine/tyrosine/threonine kinase) which results in the suppression of the ERK signaling pathway.

Therefore, SUMO and other PTMs function closely and dynamically to co-modify subsets of proteins.

Figure 21: Crosstalk of PTMs such as phosphorylation, and acetylation. A. SUMOylation can be dependent on phosphorylation (P) or acetylation; B. A wide range of PTM enzymes can be modified by SUMO which, in turn, affects their function; C. SUMO targets can be found at proteins with acetylation (Ac) and (P) targets. From (Hendriks and Vertegaal 2016)
Modification of SUMO by PTMs

SUMO family members are themselves subjected to modifications as revealed by global analyses of different PTMs. Table III displays the residues in human SUMO1, SUMO2, and SUMO3 which are modified by phosphorylation, ubiquitination, and acetylation. For instance, the binding interface for SUMO itself can be disrupted by the acetylation of SUMO itself, thus providing an additional regulatory mechanism for SUMOylation (Figure 21C) (Hendriks and Vertegaal 2016).

Table III List of modified residues in human SUMO1, SUMO2, and SUMO3 by phosphorylation, acetylation, and ubiquitination. From (Hendriks and Vertegaal 2016)

<table>
<thead>
<tr>
<th>SUMO</th>
<th>Modification</th>
<th>Modified residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>hSUMO1</td>
<td>Phosphorylation</td>
<td>Ser2, 9, and 32; Thr10, and 76</td>
</tr>
<tr>
<td></td>
<td>Acetylation</td>
<td>Lys23, and 37</td>
</tr>
<tr>
<td></td>
<td>Ubiquitination</td>
<td>Lys7, 17, 23, 25, 37, 39, 48, and 78.</td>
</tr>
<tr>
<td>hSUMO2</td>
<td>Phosphorylation</td>
<td>Ser2, 27, and 53; Thr12, and 37</td>
</tr>
<tr>
<td></td>
<td>Acetylation</td>
<td>Lys11, 32, 41, and 44</td>
</tr>
<tr>
<td></td>
<td>Ubiquitination</td>
<td>Lys7, 11, 20, 32, 34, 41, and 44</td>
</tr>
<tr>
<td>hSUMO3</td>
<td>Phosphorylation</td>
<td>Ser28, and 54; Thr12, and 38</td>
</tr>
<tr>
<td></td>
<td>Acetylation</td>
<td>Lys11, 33, 42, and 45</td>
</tr>
<tr>
<td></td>
<td>Ubiquitination</td>
<td>Lys7, 11, 21, 33, 35, 42, and 45</td>
</tr>
</tbody>
</table>

Furthermore, SUMO can form mixed chains that includes SUMO1 as the distal SUMO in the chain (Figure 22A), however, mixed SUMO-ubiquitin chain formation displays a more complex picture, particularly when these mixed chains can harbor other modifications such as phosphorylation and acetylation (Figure 22B and C). In these mixed chains, SUMO could be proximal or distal, as

Figure 22: Modification of SUMO family members by other PTMs produces complex signaling codes. A. SUMO2/3 forms chains that may include SUMO1 at distal end; B. SUMO can form mixed chains with ubiquitin through ubiquitination of SUMO and SUMOylation of ubiquitin. Longer and branched chains can also exist; C. SUMO chains and mixed ubiquitin chains can be modified by phosphorylation and acetylation, further adding signaling complexity. From (Hendriks and Vertegaal 2016)
evidenced for ubiquitination of SUMO family members and SUMOylation of ubiquitin (Hendriks et al. 2014).

Finally, as Lys residues can be acetylated as well, it might be able to potentially block the formation of chains comprising SUMO and ubiquitin or multiple SUMO moieties (Figure 22C). It remains unclear whether the proteins could be targeted for degradation more efficiently by these mixed chains than by chains of ubiquitin alone. Therefore, these cross-talks of SUMO with other PTMs further increases the signaling complexity.

2.5 Molecular and Cellular Consequences of SUMOylation

SUMO proteins are indispensable for the normal function of all eukaryotic cells. SUMOylation might influence the stability, localization or activity of a target protein. At the molecular level, SUMOylation may influence interactions with other macromolecules by altering protein surfaces. Furthermore, SUMOylation regulates and has important implications in various cellular processes such as cell cycle progression, transcription, chromosome stability, protein folding, mRNA modification, etc.

2.5.1 Molecular consequences of SUMOylation

There are three non-mutually exclusive effects of protein modification by SUMO (Hendriks et al. 2014; Tammsalu et al. 2014).

First, the binding site of a substrate that interacts with the substrate protein might be masked by SUMOylation which occludes the interaction with its partners in a SUMOylation-dependent manner. For example, the interaction of the ubiquitin-conjugating enzyme E2-25k with the ubiquitin E1 enzyme is inhibited by the SUMOylation of E2-25k which results in a decrease in its capability to conjugate ubiquitin to substrate proteins (Figure 23a) (Pichler et al. 2005).

Second, in contrast, SUMO might act as an interaction hub that recruits new interacting proteins to the substrate (Wilkinson and Henley 2010). This might be either by a direct non-covalent interaction with the SUMO moiety or via a new interaction domain created at the SUMO-substrate interface (Figure 23b). For example, the interaction of RanBP2 with RanGAP1 is promoted by the SUMOylation of RanGAP1. Further, the SUMOylation of RanGAP1 relocates it from the cytosol to the nuclear pore (Matunis, Coutavas, and Blobel 1996; Rohit Mahajan et al. 1997).

Thirdly, SUMOylation can cause conformational changes in the SUMOylated substrate, thereby, regulating its function directly (Fig 23c). For example, X-ray structure and biochemical analysis of a SUMOylated TDG fragment revealed that it undergoes conformational changes upon binding of SUMO1 which results in a loss of DNA binding (Hardeland et al. 2002; Maiti et al. 2008). This change in the conformation is mediated by an interaction of covalently attached SUMO with a non-covalently SUMO-binding site in TDG (Geiss-Friedlander and Melchior 2007).
**Genome Stability**

The genome integrity is often endangered by the internal and external factors that damage DNA and interfere with DNA replication such as DNA-damaging chemicals, ionizing radiation, UV light, and spontaneous errors that occur during DNA replication. The DNA damage response, an integrated and well-coordinated cellular response aimed at maintaining homeostasis, is activated by the cell in response to DNA damage. The DNA damage response includes cell cycle arrest, rewiring of transcriptional programs, and activation of DNA repair mechanisms (Santocanale and Diffley 1998; Lopes et al. 2001; Lisby et al. 2004; Enserink et al. 2006; Dotiwala et al. 2007).

The best studied SUMO-targets in maintenance of genome stability is proliferating cell nuclear antigen (PCNA). PCNA is modified at K164 and to a lesser extent at K127 during unperturbed DNA replication. PCNA’s SUMOylation results in recruitment of the helicase Srs2 which helps in the prevention of unscheduled homologous recombination (N. Garcia-Rodriguez, Wong, and Ulrich 2016). Therefore, SUMOylation of multiple proteins in the same pathway leads to the stability of the complexes to facilitate efficient DNA repair.

**Regulation of the cell cycle**

SUMO plays an important role in regulating the cell cycle by targeting several critical proteins including cyclin dependent kinases (CDKs) such as Cdk1, Cdk2, and Cdk6 (Eifler and Vertegaal...
Furthermore, the SUMO E2 Ubc9, several E3 ligases including PIASγ and RanBP2, and SUMO proteases SENP1 and SENP2, are required in the efficient formation of the mitotic spindle and progression through mitosis. Together, these proteins control the SUMOylation of a large group of proteins required for mitotic spindle dynamics (Eifler and Vertegaal 2015). Therefore, via controlling the regulation of key players in the cell cycle, SUMOylation participates in different phases of the cell cycle (Enserink 2017).

**Transcription**

Several studies in yeast and humans have established that SUMOylation targets several transcription factors (TFs) and chromatin regulators (Chymkowitch, Ngua P, and Enserink 2015; Enserink 2015). Owing to these studies, SUMOylation has been perceived to be a transcriptional repressor. For instance, in mammalian cells, the degradation of the IκBα (inhibitor of the transcription factor NFκB) is prevented by SUMOylation which ultimately results in the suppression of NFκB gene transcription. In addition, SUMOylation also targets several transcription co-regulators. For example, the interaction of p300 (also referred to as histone acetyl transferase p300 HAT) with HDAC6 is promoted by the SUMOylation of p300 which counteracts the positive effects of p300 on transcription.

Interestingly, SUMOylation can directly inhibit the transactivation activity of TFs. For example, SUMOylation of PPARα represses transcription of PPARα-target genes by serving as a docking site for the interaction with the nuclear receptor co-repressor (NCoR) (Ghisletti et al. 2007).

In contrast to the role of SUMO as a transcription repressor, studies have demonstrated that SUMO can stimulate the transcription of its targets as well. For instance, the association of Rap1 (yeast transcription factor) and TFIID is promoted by the SUMOylation of Rap1. The outcome is the increase in expression of its target genes which results from the recruitment of the basal transcription machinery to Rap1-regulated promoters. However, the physiological relevance of SUMOylation of the majority of TFs and chromatin-modifying enzymes remains to be established (Chymkowitch et al. 2015).

**RNA Editing and mRNA translation regulation**

The enzyme ADAR1 (adenosine deaminase that acts on RNA) is targeted by SUMOylation. They interact with Dicer to promote microRNA processing and hence, modify miRNAs. ADAR1 is SUMOylated on IK4i8LE and its SUMOylation results in reduction of its editing activity (C. Song et al. 2016). On the other hand, the efficiency of translation is regulated by SUMO at multiple levels. For example, the synthesis of tRNA (transfer RNA) as well as the transcription and maturation of ribosome components and ribosome biogenesis factors are promoted by SUMOylation. In mammalian cells, eIF4E, the key eukaryotic translation initiation factor 4E, is SUMOylated on K36, 49, 162, 206, and 212. Interestingly, the expression of unSUMOylatable eIF4E impairs the translation efficiency. Altogether, SUMOylation contributes by regulating critical processes in ribosome biogenesis (X. Xu et al. 2010).
2.6 SUMO in Disease

SUMOylation is a critical post-translational modification and is involved in the regulation of various cellular processes as discussed above. As an obvious consequence, SUMOylation plays an important role in disease progression (Flotho and Melchior 2013).

2.6.1 SUMO in Neurodegenerate Diseases

**Parkinson’s Disease**

Parkinson’s disease (PD) is a common neurodegenerative disorder and α-synuclein plays an important role in this pathology (Corti, Lesage, and Brice 2011; Stefanis 2012). Several proteins that are associated with PD have been linked with SUMO. A study suggested that α-synuclein is a target of SUMO1 and this raised a question about the identity of SUMO targets within the aggregates (Krumova et al. 2011).

Furthermore, another protein which interacts non-covalently with SUMO1 in vitro and in vivo is the E3 ubiquitin ligase, Parkin. Indeed, mutations in parkin are associated with many of the familial cases of PD. It was demonstrated that parkin ubiquitinates and degrades the SUMO E3 ligase, RanBP2. In a negative feedback loop, the interaction of SUMO1 and parkin results in the enhancement of the ubiquitin ligase activity of parkin (Um and Chung 2006).

Finally, SUMO targets DJ-1, a protein involved in the transcriptional regulation of a variety of genes involved in the cellular response to oxidative stress. Interestingly, loss of function of DJ-1 is associated with the onset of PD and has been shown to interact with SUMO E3 ligases. Two mutants of DJ-1, K130 and L166P, signify the involvement of SUMO pathway in PD. Mutation of K130, the acceptor lysine, abolishes all known functions of DJ-1 in cultured cells whereas L166P (a disease associated missense mutation) has been shown to have increased SUMOylation levels compared to the wild-type DJ-1. The authors suggested a possibility that the improper SUMOylation of the L166P mutant might promote aggregation which causes the observed increase in protein insolubility further leading to protein degradation (Shinbo et al. 2006; N. Zhong and Xu 2008).

**Alzheimer’s disease**

Alzheimer’s disease (AD) is an age-dependent neurodegenerative disorder and is the cause of the most common type of dementia associated with progressive cognitive dysfunction. The extracellular accumulation of amyloid-β (Aβ) peptides cleaved from the amyloid precursor protein (APP) by β- and γ-secretases results in the formation of these plaques. Interestingly, through an in vitro expression cloning screen, APP was thought to be a substrate of SUMO and recently it has been suggested that it can be modified on two lysine residues by both SUMO1 and SUMO2/3 in cultured cells and brain (Wolfe 2006). It was also shown that the Aβ production is negatively regulated by the SUMOylation of APP or by the overexpression of Ubc9.
Tau is involved in the stabilization of microtubules and regulation of axonal development, which are negatively regulated by its phosphorylation state. Interestingly, tau is targeted by both SUMO1 and ubiquitin. Intriguingly, it has been suggested that ubiquitin and SUMO might compete for the regulation of tau stability as upon proteasome inhibition in cultured cells there was an increase in the ubiquitination of tau and a decrease in the SUMOylation of tau (Gocke, Yu, and Kang 2005).

Figure 24 (Anderson et al. 2017) represents the different potential mechanisms of SUMOylation in neurodegenerative diseases. For example, first, the competition between SUMO and ubiquitin can modulate protein levels and affect aggregation. Second, SUMOylation of target proteins can cause sequestering of proteins to abolish critical cellular function or promote aggregation. Thirdly, nuclear body formation leads to proteosome-mediated degradation.

**SUMO and Cancer**

Studies suggest that the expression of SUMO E1 activating enzyme (a hetero-dimer SAE1 and SAE2), the E2 conjugating enzyme (Ubc9) and the SUMO E3 ligases are enhanced in numerous cancers. Table IV displays the deregulations in gene expression and locations of SUMO conjugation system in cancer (J. S. Lee, Choi, and Baek 2017).

Additionally, the knockdown of SUMO E1 or SUMO E2 inhibits the maintenance and self-renewal of colorectal cancer stem cells (Sujashvili 2016). On the other hand, SUMOylation of MAFB, a transcription factor involved in the regulation of lineage-specific hematopoiesis, promotes colorectal cancer tumorigenesis through the regulation of cell cycle (L.-S. Yang et al. 2016). Similarly, SUMOylation of Akt, a serine/threonine kinase, on the major acceptor site K276 is required for the cell growth and tumorigenesis (R. Li et al. 2013).
Table IV: Deregulations in gene expression and locations of SUMO conjugation system in cancer. From (Lee et al., 2017)

<table>
<thead>
<tr>
<th>Deregulation</th>
<th>Type of deregulation</th>
<th>Tumour type/disease</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMO1</td>
<td>Upregulation</td>
<td>Anaplastic large-cell lymphoma</td>
<td>Villalva et al 2002</td>
</tr>
<tr>
<td>SUMO2, UBA2</td>
<td>Upregulation</td>
<td>Hepatocellular carcinoma</td>
<td>Lee and Thorgeirsson et al 2004</td>
</tr>
<tr>
<td>Ubc9</td>
<td>Upregulation</td>
<td>Ovarian tumour; Human lung adenocarcinomas</td>
<td>Mo et al 2005</td>
</tr>
<tr>
<td>PIAS3</td>
<td>Upregulation</td>
<td>Lung, breast, prostate, colon, rectum, and brain tumour</td>
<td>Wang and Banerjee et al., 2004</td>
</tr>
<tr>
<td>PIASγ</td>
<td>Downregulation</td>
<td>Myelodysplastic syndrome</td>
<td>Veda et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Upregulation</td>
<td>Thyroid oncocytic adenoma</td>
<td>Jacques et al 2005</td>
</tr>
<tr>
<td>SENP1</td>
<td>Downregulation</td>
<td>Metastatic cancer cell line, LNCaP</td>
<td>Kim et al 2006</td>
</tr>
<tr>
<td>PML-RARα</td>
<td>Chromosomal translocation</td>
<td>Amyloid Precursor Leukaemia</td>
<td>Takahashi et al 2003; Wood et al 2003</td>
</tr>
</tbody>
</table>
Chapter 3  TRIM proteins

The TRIM family is defined as a family of proteins containing TRIpartite Motif (TRIM) (Meroni 2012a) which is composed of a RING domain (R), one or two B-Box domains (B) and a Coiled-coil domain (CC) (Reymond et al. 2001), hence, it is sometimes referred to as RBCC family. TRIM proteins also contain a highly variable C-terminal domain (Reymond et al. 2001) (Figure 25). TRIM family of proteins represents one of the largest classes of RING domain containing E3 ubiquitin ligases (Meroni 2012a) and it is the RING domain that is responsible for the E3 ubiquitin ligase activity. Interestingly, RING domain can mediate conjugation of proteins to ubiquitin (Esposito, Koliopoulos, and Rittinger 2017) or SUMO (Chu and Yang 2011) or NEDD8 (Noguchi et al. 2011).

TRIM family consists of more than 70 members known in humans and mice which can be classified into two evolutionary groups: the first group where the ancestor members are present in invertebrate species and is highly conserved throughout evolution; and the second group (younger and fast evolving) which is composed of TRIM genes present only in vertebrates (Sardiello et al. 2008). TRIM proteins self-associate through their coiled coil domains resulting in the formation of higher order complexes. They are also known to define sub-cellular compartments (Reymond et al. 2001a).

![Figure 25: Protein domain organization of TRIM proteins. From (Esposito, Koliopoulos, and Rittinger 2017)](image)

TRIM proteins have been associated with various biochemical, physiological, and pathological roles (Meroni and Diez-Roux 2005a; Ozato et al. 2008; Watanabe and Hatakeyama 2017). They are implicated in various cellular processes such as apoptosis, cell cycle progression, autophagy, viral response, innate immunity, neurogenesis, oncogenesis, pluripotency, etc. (Valentina V. Nenasheva and Tarantul 2020). Consequently, the alterations of TRIM genes are associated with many diseases such as cancer, autoimmune diseases, neurological disorders, developmental disorders, etc. (Watanabe and Hatakeyama 2017).
In this chapter, I will describe in detail about the TRIM family, their role as E3 ubiquitin ligases, their structural aspects, their molecular and cellular roles and how they are implicated in diseases. Of the known TRIM family members, owing to my research interest, I will describe specially Trim17 and Trim39 in more details.

Figure 26: Classification of different TRIM protein family members denoted C-I to C-IX with variable CTDs and numbers represents each TRIM member. A. Represents RBCC family and B. Represents RING-less members. From (Williams et al. 2019)
3.1 The Tripartite Motif

3.1.1 RING domain and its structural aspects

The RING motif was discovered in Ring1 (Really Interesting New Gene I) and is not a restrictive feature of TRIM proteins as it can be found in other proteins. A regular arrangement of cysteine (Cys) and histidine (His) residues that coordinate two zinc atoms defines the RING domain (Freemont 1993). The consensus sequence is as follows: Cys-X2-Cys-X9-39-Cys-X1-3-His-X2-3-Cys-X2-Cys-X4-48-Cys-X2-Cys (where X is any amino acid) [Figure  27 (a)] (Deshaies and Joazeiro 2009). The RING domain can be further sub-classified into the H2 type and the C2 type (Freemont 2000). The TRIM/RBCC family belongs to the C2 type which has a Cys residue in the fifth coordination site (Meroni and Diez-Roux 2005a).

It was revealed using the three-dimensional structure of the RING domain that the conserved Cysteine and Histidine residues are buried within the domains core [Figure 27(b)]. These conserved residues help maintain the overall structure through binding two atoms of zinc. Interestingly, additional semi-conserved residues are implicated either in forming the domains hydrophobic core or in recruiting other proteins. Importantly, unlike zinc fingers, the zinc coordination sites in a RING “finger” are interleaved which results in a rigid, globular platform for protein-protein interaction, hence RING domain (Barlow et al. 1994; Borden et al. 1995).

The RING domain plays a critical role in the ubiquitination process. It binds to ubiquitin conjugating enzymes, E2, and promotes the transfer of ubiquitin to the substrates (Joazeiro and Weissman 2000). Therefore, it serves as a scaffold that brings E2 and substrate together. The bioinformatic analysis revealed that ~300 human genes encode RING domain proteins. Majority of the proteins are involved in the ubiquitination pathway as the RING domain proteins mainly function as an E3 ubiquitin-
ligases (Joazeiro and Weissman 2000; Xing Li and Sodroski 2008). Importantly, TRIM proteins are E3 ubiquitin-ligases except 8 members (TRIM14, 16, 20, 29, 44, 66, 70, and 76) that lack the RING domain (Meroni and Diez-Roux 2005b) but share the same domain composition in other respects. Another key feature of the RING domain is its participation in homo and heterodimerization (Bellon et al. 1997; Brzovic et al. 2001).

3.1.2 B-Box domains and its structural aspects

The B-Box domains belong to the category of zinc-finger domains and typically contain cysteine and histidine residues arranged in one of the several motifs which are relatively conserved. B-Box domains are a crucial feature of TRIM proteins, however, can be found in other protein families. They are composed of B1 and B2 domains, however, some TRIM proteins only contain a B2 domain (eg.TRIM13, 20, 29, 37, 44, 55, 59, 63, 76) (Micale et al. 2012). B-Boxes may contain two different zinc binding motifs whose zinc-binding consensus sequence is as follows (Reymond et al. 2001a; Michael A. Massiah et al. 2007):

B-Box1: C-X(2)-C-X(6-17)-C-X(2)-C-X(4-8)-C-X(2-3)-C/H-X(3-4)-H-X(5-10)-H [C5(C/H)H2]

B-Box2: C-X(2-4)-H-X(7-10)-C-X(1-4)-D/C(4-7)-C-X(2)-C-X(3-6)-H-X(2-5)-H [CHC(D/C)C2H2]

The first structural studies that enabled us to gain an insight into B-Box domains were based on TRIM18/MID1 protein (Michael A. Massiah et al. 2006; 2007). Using nuclear magnetic resonance (NMR) spectroscopy, the structure of B-Box1 domain revealed that it coordinates two zinc ions in a cross-brace manner with six cysteine and two histidine residues. It consists of three turn α-helix, two short β-strands and three β-turns which binds two zinc atoms and encompasses Valine 117 to Proline 164 (Figure 28) (Michael A. Massiah et al. 2006). The structure of B-Box2 domain of MID1 consists of seven classical Cys and His zinc binding residues which suggests that only one zinc might be coordinated by four of these residues (Figure 28). Basically, the B-Box2 domain adopts a two-turn α-helix, two short β-strands separated by a type-2 β-turn, and two structural loops adjacent to the helix (Michael Anthony Massiah 2019). Till date, the structure of B-Box1 and B-Box2 domains has been solved for several TRIM proteins and suggest that all B-Box domain structures are similar. Even though mutations in some B-Boxes have been linked to disease phenotypes, such as P130S in TRIM32 with Bardet-Biedl syndrome or C142S and C145Y in TRIM18 with XLOS, no specific functional role in the context of full-length protein has been assigned to the B-Box domains. Initial studies suggested that B-Box1 might facilitate the interaction with cognate E2 while B-Box2 might help in the regulation of catalytic activity (Michael A. Massiah et al. 2006). Studies done on TRIM5α, however, highlight that B-boxes can self-associate and mediate protein-protein interactions pointing its role as molecular scaffolds where it promotes higher order oligomerisation or might contribute to substrate recruitment or even restrict the conformational space available to the RING domain by acting as molecular spacers or pins (Esposito, Koliopoulos, and Rittinger 2017).
Coiled-coil domains and their structural aspects

The coiled-coil motifs can be found in numerous proteins where they have diverse functions (J. Liu et al. 2006). The coiled-coil motif of TRIM proteins is around 100 residues long and is often broken up into two or three separate coiled-coil motifs. A ‘rope-like structure’ stabilized by hydrophobic interactions is formed by alpha helices that are wound together in coiled-coil domains. Notably, the primary sequence of this region is not conserved (Micale et al. 2012).

The coiled-coil domain is mainly involved in homo and hetero-interactions and in the promotion of high molecular weight complexes (Napolitano and Meroni 2012a). Using interaction-mating technique, Reymond and colleagues showed that a consistent number of TRIM members were able to homo-interact whereas heterologous interactions were rare. This suggested that homooligomerization had a high degree of specificity. However, it was demonstrated that TRIM44 and TRIM17 (Terf) can hetero-dimerize and this resulted in the regulation of the ubiquitination and stabilization of the latter. Results of co-immunoprecipitation assays using TRIM44 and the deletion mutants of Terf suggested that indeed it’s the CC domain that sustains these protein-protein interactions (Urano et al. 2009). Other examples of hetero-interactions have been also reported.

3.1.3 Coiled-coil domains and their structural aspects

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Figure 28: Structure of B-Box domains of MID1. A. Represents the cross-brace zinc-binding mechanism by Cys/His-rich sequences of B Box domains. B. Ribbon drawings of B-Box1 and B-Box2 domains of MID1. From (Michael Anthony Massiah 2019)
TRIM protein mutants carrying individual deletions of relevant protein regions were analysed using interaction mating and co-immunoprecipitation to map the structural determinant responsible for their self-association. Indeed, it was the deletion of the CC region that resulted in the loss of self-association whereas the binding was partially affected when other regions were deleted. Interestingly, self-association was observed with isolated CC, but not R or BB, suggesting that the CC region is necessary and sufficient for homo-interaction. In the same work, it appeared that the ability of TRIM proteins to form higher molecular weight complexes and to define discrete subcellular compartments within the cell is the consequence of the self-association of the CC (Reymond et al. 2001b) (Napolitano and Meroni 2012).

3.1.4 C-Terminal domains and their structural aspects

TRIM proteins can be classified into 11 subfamilies based on their C-Terminal domains (CTDs) (Figure 26). CTDs, which sometimes appear alone or in combination with other domains, may mediate substrate recognition and regulate protein-protein interactions (Ozato et al. 2008; Reymond et al. 2001a). The PRY-SPRY domain accounts for the majority of CTDs as it is present in two-thirds of the TRIM proteins, notably TRIM17 and TRIM39, hence only PRY-SPRY domain is the focus of this chapter. The second most common CTD found is NHL (named after the identification in NCL-1, HT2A, and LIN-41 proteins). It consists of five or six repeats that fold into a barrel-like β-propeller structure and it aids in mediating protein and RNA interactions. Another domain which can be observed is tandem plant homeodomain and bromodomain (PHD, BR) which characterize the transcriptional intermediary factor (TIF1) subgroup of TRIM family and is involved in chromatin binding. The other domains such as Meprin and TRAF-homology domain (MATH), ADP-ribosylation factor (ARF) family domain, C-terminal subgroup one signature (COS), filamin-type immunoglobulin (FIL), fibronectin type 3 repeat (FN3), and transmembrane (TM) region are also found, however, they are less represented compared to the above mentioned CTDs (Rienzo et al. 2020).

3.1.5 PRY-SPRY domains

The B30.2 domain (also known as RFP-like or PRYSPRY) was originally identified based on sequence homology to a protein encoded by the B30.2 exon which was located within the major histocompatibility complex (MHC) Class I region and was later defined by the presence of three highly conserved sequence motifs (LDE, WEVE, and, LDYE). Interestingly, the SPRY domain was identified based on a sequence repeat in the dual specificity kinase spore lysis A found in Dictostelium discoideum and in all three mammalian Ca²⁺ release channel ryanodine receptors (RyR). In many cases, N-terminal to this domain is the SPRY-associated (PRY) region that extends the domain to form a PRY/SPRY fusion (D’Cruz et al. 2013). It contains -61-amino-acids for PRY and -140-amino-acid for SPRY domains. 11 different human protein families contain these domains, however, they are most prominent in human and mouse TRIM family members (Woo et al. 2006; Grütter et al. 2006). The PRY-SPRY domains (also referred to as B30.2) are found in 24 family members but the SPRY domain alone is found in 39 human TRIM family members (Rhodes, de
Bono, and Trowsdale 2005). The PRY-SPRY domain core is formed into a distorted two-layer β-sandwich with anti-parallel arrangement of β strands, which tend to be stable in length among species.

The role of B30.2 domain in the TRIM family can be evidenced in the regulation of immune response to retroviruses. For example, TRIM5α is a cellular restriction factor for infection by HIV-1 on rhesus monkey cells (Stremlau et al. 2004; Goldschmidt et al. 2008). Besides TRIM5α, most TRIM proteins which have been reported in the antiviral context such as TRIM1, 22, 26, and 34, possess a B30.2 domain at the C-terminus (Micale et al. 2012). Interestingly, the PRY-SPRY domain has an influence in the subcellular localization of some TRIM proteins. For instance, the PRY-SPRY domain of TRIM22 protein is critical to localize the protein in distinct nuclear bodies (Sivaramakrishnan et al. 2009; Herr, Dressel, and Walter 2009). Similarly, changes in the localization of TRIM9 and TRIM18 were observed as the mutations in their PRY-SPRY domain changed their localization from microtubule-associated structures to cytoplasmic speckles (Short and Cox 2006).

Mutations in PRY-SPRY domains are associated with different diseases. For example, a TRIM41 variant “p.R534C”, was found to co-segregate with Parkinson’s disease. Interestingly, the R534C substitution is located in the PRY-SPRY domain of TRIM41 in PD patients. It was demonstrated using co-immunoprecipitation that the binding of TRIM41 p.R534C mutant to its substrate ZSCAN21 was reduced by ~50% compared to the WT-TRIM41 (Irëna Lassot et al. 2018). ZSCAN21 is a transcription factor that favours the expression of α-synuclein. Therefore, this variation in the PRY-SPRY domain of TRIM41 may cause reduced ubiquitination and degradation of ZSCAN21 resulting in increased α-synuclein, a protein whose accumulation plays a crucial role in PD.

3.2 Functional and Structural aspects of TRIM proteins

3.2.1 Interactions between ubiquitin E2 enzymes and TRIM proteins

Understanding the specific interaction between TRIM proteins as E3 ubiquitin ligases and their cognate E2s is an important aspect which might help to determine the fate of their targets. Napolitano et al., studied the relationship between the E2 enzymes and the TRIM proteins. In their study, they tested the interaction between 42 TRIM proteins and 26 best studied UBE2 enzymes using a yeast two-hybrid system. Of the E2 families tested, most of the TRIM proteins had preference for the UBE2 belonging to the type D and E family. Importantly, different TRIM protein members have a tendency to interact with common E2s. For example, the E2’s shared by TRIM17 and TRIM39 include UBE2D1, UBE2D2, UBE2D3, UBE2D4, UBE2H, and UBE2W. However, some exclusive interactions were observed, for example, between TRIM9 and UBE2G2. Results suggest that majority of TRIM proteins directly interact with selected UBE2 enzymes via their RING domain. An interesting observation from these studies was that in the absence of a specific substrate, the predominant reaction catalysed was auto-ubiquitination (Napolitano et al. 2011).
3.2.2 Homo and hetero-interactions between TRIM proteins

**Homo-interactions between TRIMs**

The ability of TRIM proteins to homo-interact through their CC region is one of their main structural feature (Reymond et al. 2001). Using interaction mating technique and *in-vitro* and or *in-vivo* co-immunoprecipitation techniques, they confirmed homo-interactions of TRIM1, 3, 5, 6, 8, 9, 10, 11, 18, 21, 23, 24, 25, 26, 27, 29, 30, 31, and 32. Interestingly, for several TRIM members such as TRIM6, 8, 11, 18, 19, 23, 28, 29, and 30 higher molecular weight homocomplexes compatible with the presence of more than two TRIM molecules have also been observed. Altogether, these results suggest that the ability of different TRIM proteins to form higher molecular weight complexes is the consequence of the self-association properties of their CC regions (Reymond et al. 2001b).

![Diagram](image)

**Figure 29**: (A) Different proposed models for possible TRIM homocomplexes on the E3 activity. The complexes are represented as dimers whereas the colours grey and orange represent two different E2 enzymes. A. Homocomplex of TRIMs. (B) Different proposed models for possible TRIM heterocomplexes effects on the E3 activity. B. Heterocomplex of TRIMs: upper drawing represents interaction with the same E2 enzyme and the lower drawing represents interaction with different E2 enzymes. From (Napolitano and Meroni 2012). (C) Heterocomplex formed by a TRIM protein and a RING-less TRIM protein. From (Napolitano and Meroni 2012)
Despite, the available data on the structural arrangement of dimers, it is not clear whether this homodimerization is required for the TRIM E3 ligase activity. One possibility is that the homocomplex is required for substrate binding, and each TRIM molecule within the homo-dimeric structure might interact with either the same or different E2 enzymes which ultimately influences the dynamicity, processivity, and topology of the chain attached to the target [Figure 29(A)] (Napolitano and Meroni 2012a).

**Hetero-interactions between TRIMs**

TRIM proteins can also hetero-interact. For example, TRIM21 interacts with TRIM5 leading to the ubiquitination and degradation of the latter in HEK293 cells (Yamauchi et al. 2008). This E3-substrate relationship between two TRIM proteins might be true for other TRIM heterointeractions either reported or postulated such as those involving TRIM5α and a group of TRIM proteins (TRIM4, 6, 22, 27, and 34) that interestingly show colocalization within cytoplasmic bodies (Xing Li et al. 2007).

Importantly, recent studies in our group reveal heterointeractions between different TRIM proteins such as TRIM17, TRIM28, and TRIM41. Using co-immunoprecipitation technique, Lionnard et al., demonstrated that ectopically expressed TRIM17 and TRIM28 proteins interact with each other. Interestingly, specific PLA signal were also observed between endogenous TRIM17 and TRIM28 proteins in SK-MEL-28 cells (Lionnard et al. 2019). Furthermore, in a separate study, Lassot et al., demonstrated that TRIM17 and TRIM41 heterointeract with each other. In a Y2H screen, TRIM41 was identified as a putative partner of TRIM17 and indeed their ability to hetero-interact with each other was confirmed using co-immunoprecipitation (Irëna Lassot et al. 2018). Importantly, the interaction leads to the inhibition of E3 activity of TRIM28 or TRIM41 by TRIM17. Notably, the hetero-interactions of TRIM17 with TRIM28 or TRIM41 does not lead to ubiquitination or degradation in contrast to others mentioned above.

Interestingly, what is largely missing for most hetero-interactions is the exact composition of the complexes and the consequence of such interactions on the E3 ligase activity. Similar to TRIM homo-interactions, the single E3 activity is predicted to be further diversified, thereby, increasing the possibility to be regulated depending on the context [Figure 29 (B)] (Napolitano and Meroni 2012a).

Heterointeractions have also been detected between a regular TRIM and a RING-less TRIM protein. A RING-less TRIM44 has been shown to bind and stabilize TRIM17/Terf. This occurs through a deubiquitinating activity present in the N-terminal ubiquitin carboxyl-terminal hydrolase the zinc finger (ZF-UBP) domain of TRIM44 (Urano et al. 2009). It has also been speculated that this type of cross-regulation may apply to other TRIM-containing heterocomplexes especially involving RING-less TRIM proteins [Figure 29(C)]. For instance, levels of Tip60 and β-catenin might be regulated by another RING-less TRIM29 through a heterocomplex formation with a canonical TRIM protein.
3.3 Molecular and cellular functions of TRIMs

3.3.1 Molecular functions of TRIMs

**TRIM proteins as E3 ubiquitin ligases**

As mentioned above, E3 enzymes play a crucial role in specifically recognizing the substrate. It has been shown that TRIM proteins act as E3 ubiquitin ligases (Meroni and Diez-Roux 2005a) and that their RING domain is responsible for this activity. The dimerization of the RING domain seems to be the pre-requisite for its catalytic activity (Koliopoulos et al. 2016). Other domains such as B-Box are structurally related to RING domain and in some cases might contribute by amplifying the RING domain E3 activity (Han, Du, and Massiah 2011). It is interesting to note that the interaction of TRIM proteins as E3’s with their E2’s plays an equally important role in the fate of the target protein as this interaction might determine the type of chain formation (Deshaies and Joazeiro 2009; Ye and Rape 2009).

Several members of the TRIM family have been demonstrated to act as E3 ubiquitin ligases, however, the function of most of the members of this family remains unclear (Kazuhiro Ikeda and Inoue 2012). Table V shows the list of few TRIM members that have been identified as E3s alongside their functions.

Altogether, TRIM proteins acting as E3 ligases indicate their importance in regulate different cellular functions. Indeed, it is crucial to further investigate their capacity in therapeutics.

Table V : List of few E3 ubiquitin ligases in TRIM proteins and their function. From (Kazuhiro Ikeda and Inoue 2012).

<table>
<thead>
<tr>
<th>TRIM Family Member</th>
<th>Substrate</th>
<th>Function</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 TRIM5</td>
<td>HIV-1 gag proteins</td>
<td>TRIM5 is essential for retroviral restriction; it targets incoming retroviral targets incoming retroviral capsids after viral penetration or Gag assembly during the production of HIV-1</td>
<td>(Sakuma et al. 2007)</td>
</tr>
<tr>
<td>2 TRIM11</td>
<td>Humanin</td>
<td>Is known to suppress Alzheimer’s disease related neurotoxicity. It exerts anti-apoptotic effects by interfering with Bax function.</td>
<td>(Niikura et al. 2003)</td>
</tr>
<tr>
<td>3 Trim17/Terf</td>
<td>Mcl-1</td>
<td>The ubiquitination and degradation of the anti-apoptotic protein Mcl-1 mediated by Trim17 is necessary for initiating neuronal death</td>
<td>(Magiera et al. 2013a)</td>
</tr>
<tr>
<td>4</td>
<td>TRIM18/MID1</td>
<td>PP2A</td>
<td>MID1 is involved in the developmental Opitz G/BBB syndrome. It plays a physiological role in microtubule dynamics and is identified as an autoantigen in Sjögren syndrome. TRIM21 plays a significant role in the quality control of IgG1 heavy chain; IRF-8</td>
</tr>
<tr>
<td>5</td>
<td>TRIM21</td>
<td>human IgG1 heavy chain; IRF-8</td>
<td>-by ubiquitinating IRF-8, it enhances the expression of IL-12p40 in IFN-γ/TLR-stimulated macrophages. Therefore, TRIM21 contributes to innate immunity</td>
</tr>
<tr>
<td>6</td>
<td>TRIM28</td>
<td>BCL2A1</td>
<td>TRIM28 reduces the protein levels of the anti-apoptotic protein BCL2A1, thereby restoring the sensitivity of melanoma cells to BRAF-targeted therapy.</td>
</tr>
<tr>
<td>7</td>
<td>TRIM32</td>
<td>actin</td>
<td>TRIM32 participates in myofibrillar protein turnover, especially during muscle adaptation</td>
</tr>
<tr>
<td>8</td>
<td>TRIM39</td>
<td>p53</td>
<td>The knockdown of Trim39 1) induces G1/S arrest, thereby, inhibiting cell proliferation; 2) enhances cell killing by nutlin-3a (inhibitor of MDM2, a known E3 ligase of p53), therefore increasing the efficacy of nutlin-3a in killing p53-positive cancer cells. By inducing the degradation of the transcription factor ZSCAN21, TRIM41 modulates the expression of α-synuclein whose accumulation is crucial for Parkinson disease.</td>
</tr>
<tr>
<td>9</td>
<td>TRIM41</td>
<td>ZSCAN21</td>
<td>MuRF1 might play a critical role in muscle turnover as MuRF1/−/+ exhibited increased resistance to muscular atrophy and significant muscle preservation after denervation.</td>
</tr>
</tbody>
</table>
| 10 | TRIM63/MuRF1 | Troponin-1 | TRIM proteins as SUMO E3 ligases
As mentioned in the previous chapter, numerous cellular processes are governed by SUMOylation, however, there are only limited number of SUMO protein ligases (E3s) which have been identified so far. Intriguingly, one study showed that some members of the TRIM family such as Promyelocytic | (Robin, Opitz, and Muenke 1996; Quaderi et al. 1997; Short et al. 2002) |

TRIM proteins as SUMO E3 ligases

As mentioned in the previous chapter, numerous cellular processes are governed by SUMOylation, however, there are only limited number of SUMO protein ligases (E3s) which have been identified so far. Intriguingly, one study showed that some members of the TRIM family such as Promyelocytic
leukaemia protein (PML)/TRIM19, and TRIM27 can act as SUMO E3s. It was observed that these TRIM proteins can bind to both the SUMO-conjugating enzyme Ubc9 and substrates. Consequently, the transfer of SUMOs from Ubc9 to these substrates was strongly enhanced by these TRIM SUMO E3s (Chu and Yang 2011).

It is known that SUMO modifies PML and the overexpression of PML enhanced overall SUMOylation in yeast cells (Kamitani et al. 1998; S. Müller et al. 2000; Quimby et al. 2006). The in-vitro and in-vivo experiments confirmed that PML stimulated the SUMOylation of p53. Interestingly, experiments also supported that PML increased the conjugation of both SUMO1 and SUMO2 to Mdm2 (a ubiquitin ligase of p53) and of SUMO1 to c-Jun (a transcription factor). Therefore, it was clear that the SUMOylation of multiple nuclear proteins was enhanced by PML and that the likely required structural determinants in PML were its RING domain and the B-Box domains (Chu and Yang 2011).

The group also screened 14 other TRIM proteins from various subgroups. Observed data suggested that half of these TRIM proteins enhanced the SUMOylation of Mdm2. Interestingly, TRIM27 and TRIM32 exhibited higher activity in stimulating the SUMOylation of Mdm2 compared to PML (Chu and Yang 2011), however, these observations need further investigation.

**RNA-binding roles in the TRIM family**

It has only recently emerged that many TRIM proteins might be involved in direct RNA binding via their C-terminal NHL or PRY/SPRY domains (Sonoda and Wharton 2001; Loedige et al. 2013). Cellular fate is governed by controlling gene products at the RNA and protein levels. Various diseases such as neurological disorders and cancer are caused by the mutations in the RNA-binding proteins (RBPs) (Cooper, Wan, and Dreyfuss 2009). Interestingly, several of the TRIM family members have been identified as RBPs. Certain TRIM proteins have been known to have RNA related functions and it is their C-terminal domains which are involved in such interactions.

The first evidence of direct RNA binding of TRIM proteins was presented for BRAT (BRAin Tumour), which is a member of the TRIM-NHL subfamily in *Drosophila* (Loedige et al. 2014). TRIM25, TRIM28, TRIM56, and TRIM71 were identified as RBPs in a mRNA interactome capture studies done in HEK293, HeLa, and mouse embryonic stem cells (mESCs) (Baltz et al. 2012; Castello et al. 2012; Kwon et al. 2013). Apart from the above listed TRIM proteins TRIM33, TRIM44 and TRIM26 were also identified in a more unbiased protein-RNA crosslinking capture (Trendel et al. 2019). Importantly, in a GST pull-down and proteomics assay in a lysate from apoptotic neurons, our group identified that Trim17 could pull-down many RNA binding proteins involved in RNA metabolism (splicing, transport, and translation) (unpublished data).

As an example, TRIM25 has been shown to interact with a variety of RNAs, including 3-UTRs and exons of mRNAs, lincRNAs, miRNAs, viral RNAs and their corresponding RNPs. In fact, TRIM25 links RNA binding and ubiquitination. Experimental evidence suggests a speculative mechanism of RNA assisted ubiquitination of RIG-1 by TRIM25. In the absence of RNA, substrate-binding PRY/SPRY domain of TRIM25 interacts transiently with its CC (Koliopoulos et al. 2018). A conformational change in TRIM25 is induced upon RNA-binding via remodulation of the linker that
connects CC and PRY/SPRY domain (Sanchez et al. 2018) leading to its activation (Kwon et al. 2013; Choudhury et al. 2017). The same RNA-strand is bound by RIG-1 (substrate of TRIM25) via its helicase and CTD (Kolakofsky, Kowalinski, and Cusack 2012). Then, the CARD domains of RIG-1 is poly-ubiquitinated by TRIM25 and this triggers RIG-1 signalling (H. Lin et al. 2019).

**TRIM proteins as transcriptional regulators**

TRIM proteins regulate the activity of various transcription factors (for eg. NFATc3, ZSCAN21, etc.) and therefore control gene expression. TRIM proteins have been described as coregulators of transcription factors in Drosophila, C. elegans and mammals (Beckstead et al. 2005; Kiefer, Smith, and Mango 2007; Le Douarin et al. 1995; Friedman et al. 1996; S. Zhong, Salomoni, and Pandolfi 2000). The precise mechanism by which TRIM proteins influence transcription is not clear, however, evidence suggest that they can have a role at the chromatin level. For eg., TIF (Transcriptional intermediary factors) family members such as TIF1α/TRIM24, TIF1β/TRIM28, and TIF1δ/TRIM66 act as chromatin-related cofactors (Le Douarin et al. 1996; Nielsen et al. 1999; Schultz, Friedman, and Rauscher 2001; Schultz et al. 2002; Khetchoumian et al. 2004). Additionally, evidence suggest that TRIM19 and TRIM27 regulate transcription by interacting with chromatin modifiers (W. S. Wu et al. 2001; Shimono et al. 2000). Table VI displays the role of a few TRIM proteins in regulating transcription.

<table>
<thead>
<tr>
<th>TRIM member</th>
<th>DNA binding Transcription Factor</th>
<th>Transcriptional effect, Mechanism of action</th>
<th>Biological function</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 TRIM24/TIF1α</td>
<td>Nuclear receptors</td>
<td>Modulates ligand-dependent transactivation by nuclear receptors</td>
<td>-Liver tumour suppression</td>
<td>(Le Douarin et al. 1995; 1996; Nielsen et al. 1999)</td>
</tr>
<tr>
<td>2 TRIM28/TIF1β</td>
<td>KRAB-ZFPs</td>
<td>Recruits chromatin modifiers, mediates KRAB-ZFP repression</td>
<td>Regulates progression of primitive endoderm (PrE) into terminally differentiated Parietal endoderm (PE) via differentiation and retrovirus silencing</td>
<td>(Le Douarin et al. 1996; Nielsen et al. 1999; Schultz, Friedman, and Rauscher 2001; Schultz et al. 2002)</td>
</tr>
<tr>
<td>3 TRIM66/TIF1δ</td>
<td>HP1</td>
<td>Involved in transcriptional repression when targeted to the DNA</td>
<td>Involved in regulating E2F1-mediated apoptosis</td>
<td>(C. Wang et al. 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Might be involved in postmeiotic germ cell gene expression</td>
<td>(Khetchoumian et al. 2004)</td>
</tr>
<tr>
<td>4</td>
<td>TRIM19/PML</td>
<td>Co-activation: RARα</td>
<td>It stabilizes CBP-RAR complex</td>
<td>Regulates growth inhibition, cellular differentiation</td>
</tr>
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<td>----</td>
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<td>-----------------------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>De-activation: NFκB</td>
<td>Interferes with the NFκB DNA binding</td>
<td>Represses A20 transcription, regulates apoptosis</td>
</tr>
<tr>
<td>5</td>
<td>TRIM27</td>
<td>ERα</td>
<td>Is known to interact directly with the C-ter Glu/Arg-rich region of the ERα repressor SAFB1</td>
<td>Involved in the positive regulation of a subset of ERα target genes in MCF-7 cells</td>
</tr>
<tr>
<td>6</td>
<td>TRIM17</td>
<td>NFATc3</td>
<td>Binds to NFATc3 and NFATc4 in the cytoplasm, and decreases the activity of both NFATc3 and NFATc4</td>
<td>is known to initiate neuronal apoptosis, and decreases the mRNA levels of endogenous BDNF (Brain-derived neurotrophic factor)-target gene of NFATc3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZSCAN21</td>
<td>Binds ZSCAN21 and stabilizes it</td>
<td>Increases the expression of its target gene, SNCA</td>
</tr>
<tr>
<td>7</td>
<td>TRIM41</td>
<td>ZSCAN21</td>
<td>Binds ZSCAN21 and degrades it</td>
<td>Decreases the expression of its target gene SNCA</td>
</tr>
</tbody>
</table>

### 3.3.2 Cellular functions of TRIM proteins

TRIM family proteins have various functions in cellular processes such as apoptosis, innate immunity, autophagy, osteogenesis, and neurogenesis. Few of the cellular processes where TRIM proteins play a critical role are described below.

![Figure 30: Different functions of TRIM proteins. From (Valentina V. Nenasheva and Tarantul 2020)](image-url)
**Apoptosis**

Apoptosis is defined as a form of programmed cell death which involves genetically determined elimination of cells. Apoptosis is a vital component of many processes including normal cell turnover, proper development and functioning of the immune system, embryonic development to name a few. The cysteine-aspartate proteases known as caspases initiates, regulates and, execute apoptosis (Fuchs and Steller 2011). The two categories of caspases include: initiator caspases, and the executioner caspases. After the detection of the cell damage, the initiator caspases (caspases 8 and 9) are activated from inactive procaspases, and they further activate the executioner caspases (caspases 3, 6, and 7) (D’Arcy 2019). Apoptosis can be initiated by one of the two pathways: intrinsic pathway and extrinsic pathway.

In the intrinsic pathway, apoptosis is induced by directly activating Caspase-3 or by cleaving bid (BH3 interacting domain death agonist) which results in the mitochondrial dysfunction and a subsequent release of cytochrome c and the activation of caspases-9 and 3. This pathway is tightly controlled by the B-cell lymphoma (BCL-2) protein family (Chipuk et al. 2010). Based on the BCL-2 homology (BH) domains, the members of the BCL-2 family fall into pro-apoptotic or anti-apoptotic subgroups. The pro-apoptotic members belong to either the multi-BH containing BAX and BAK or the BH3-only proteins (such as BID, BIM, PUMA) while the anti-apoptotic members include BCL-2, BCL-xL, BCL-B, BCL-w, MCL-1, and BCL2A1. They are the key regulators that control the release of cytochrome c and other apoptogenic factors from mitochondria (Lionnard et al. 2019).

The extrinsic pathway, also known as the death receptor pathway (DR), is initiated by the activation of death receptors, such as Fas, that have an intracellular death domain. This results in the formation of a death-inducing signalling complex in which the initiator caspase, caspase-8 is activated by its adaptor FAS-associated death domain (FADD). This results in the activation of the caspase cascade and apoptosis (D’Arcy 2019).

Dysregulation (too little or too much) of apoptosis is a factor contributing to many human diseases such as neurodegenerative diseases, autoimmune disorders, many types of cancers, etc. (Elmore 2007a). Several TRIM proteins are involved in regulatory roles where they participate in the initiation and execution of apoptosis such as TRIM13, TRIM16, Trim17, TRIM22, TRIM27 and Trim39. A brief description of the TRIM members regulating apoptosis is given below. Importantly, the roles of Trim17 and Trim39 in the regulation of apoptosis are discussed later in the chapter.

**TRIM13/RFP2** (Ret finger protein2): Joo et al., showed that the increased expression of RFP2 in cells following ionizing radiation induced apoptosis via the proteasomal degradation of MDM2 (and thereby stabilization of p53) and AKT (an inhibitor of apoptosis). This was dependent on the E3 ubiquitin ligase activity of RFP2 as the mutation in its RING domain failed to drive apoptosis compared to the WT-RFP2 (Joo et al. 2011).

**TRIM16**: The overexpression of TRIM16 has been shown to induce apoptosis in human breast cancer (MCF7) and neuroblastoma [BE(2)-C] cells by increasing the activity of caspase 2. Indeed, a
direct interaction between TRIM16 and caspase-2 proteins was observed in both cell types (P. Y. Kim et al. 2013).

**TRIM22:** Monocyte apoptosis is a key mechanism that governs the host immune responses during sepsis. TRIM22 has a high-level constitutive expression in monocytes and is known to play critical roles in antiviral response and inflammation (Obad et al. 2007; McNab et al. 2011). Interestingly, overexpression of TRIM22 was found to interfere with the clonogenic growth of monocyte cells which suggested that TRIM22 might regulate monocyte survival. Chen et al., demonstrated that lipopolysaccharides (LPS)-primed human peripheral blood monocytes with high levels of TRIM22 expression were more sensitive to apoptosis. Indeed, overexpression of TRIM22 modulated the expression and oligomerization/activation of the pro-apoptotic protein Bak. The deletion of RING domain or the SPRY-domain of TRIM22 resulted in a significant attenuation of TRIM22-mediated monocyte apoptosis and led to a decreased Bak expression and oligomerization. Moreover, in monocytes from septic patients, downregulation of TRIM22 levels and a positive correlation in Bak levels was observed. Taken together, these findings revealed that TRIM22 has a critical role in monocyte apoptosis where it regulates Bak oligomerization and might have a potential function in the pathogenesis of sepsis (C. Chen et al. 2017).

**TRIM27/RFP:** Dho et al., showed that the ectopic expression of RFP in HEK293 cells resulted in an extensive apoptosis. The expression of RFP activated Jun-N terminal kinase and p38 kinase and also increased caspase-3 like activity. Importantly, the intact RBCC moiety is indispensable for the pro-apoptotic function of RFP. Altogether, these results suggest that TRIM27/RFP plays a critical role in apoptosis, however, the exact effector molecule that interacts with RFP needs further investigation (Dho and Kwon 2003).

**Autophagy**

Autophagy is one of the major intracellular degradation systems in addition to the UPS by which cytoplasmic materials are enwrapped in double-membrane vesicles called autophagosomes and further delivered to lysosomes for degradation (Mizushima and Komatsu 2011; Morishita and Mizushima 2019). Over 40 autophagy-related genes/proteins (ATGs) play critical roles in the dynamics and processes of autophagic membranes. Intriguingly, ubiquitin has emerged as a key regulator of all molecular steps of the autophagy influx, from the nucleation of the double membrane to the shutdown of the entire process (Dikic and Elazar 2018). Diverse ubiquitin chains attach as selective labels on protein aggregates and dysfunctional organelles, therefore promoting their autophagy-dependent degradation. In the cytosol, UPS and autophagy lysosome (AL) pathways act simultaneously by sharing components of their molecular machineries, and influencing each other’s activity (Dikic 2017; Korolchuk et al. 2009; Marshall et al. 2015). Interestingly, the autophagy receptor sequestosome-1 (SQSTM1)/p62 is the principal molecule that regulates the cross-talk between the two systems. In autophagy, p62 helps in the recognition of the polyubiquitin chain and further recruits the targets into autophagosomes. The autophagy adaptor proteins such as p62 link
ubiquitin to the autophagosome by acting as a bridging molecule. Such proteins contain ubiquitin-associated (UBA)-domains which facilitates the binding to ubiquitin and an additional domain dedicated to linkage to autophagosomes such as LC3-interacting region (LIR) which facilitates adaptor protein binding to LC3. Therefore, the presence of these two domains in adaptor proteins facilitate the connection between the UPS and autophagy (Nam et al. 2017).

TRIM proteins have been shown to play a major role in the regulation of autophagy both in physiological and pathological conditions such as inflammation, tumorigenesis, muscle atrophy, and infection. TRIM proteins regulate the activity of the core autophagy machinery by acting as scaffold proteins or via ubiquitin-mediated mechanisms.

Several TRIM proteins have been classified as regulators of autophagy. For example, initial findings revealed that TRIM55 (MuRF2- a muscle specific ubiquitin ligase) interacted with p62 (autophagy receptor), therefore, establishing a relationship between autophagy and TRIM proteins (Lange et al. 2005; Perera et al. 2011; Pizon et al. 2013). Notably, an interaction between TRIM63 (MuRF1) and p62 was also observed and this interaction led to the regulation of nicotinic acetylcholine receptors turnover (Khan et al. 2014). On the other hand, TRIM21 mediates the mono-ubiquitination and the subcellular translocation of active IKKβ to autophagosomes (Niida, Tanaka, and Kamitani 2010). This resulted in the suppression of IKKβ-mediated NFκB signalling. TRIM13 is known to localize in the endoplasmic reticulum (ER) and was shown to induce autophagy during ER stress via its CC domain, and to interact with p62 (Tomar et al. 2012). TRIM17 was shown as an inhibitor of selective autophagy by Mandell et al. Following the knockdown of TRIM17, there was a reduction in the amount of p62 in HeLa cells. Further, treatment of these cells with an inhibitor of autophagosome maturation (bafilomycin A1), rescued p62 protein levels indicating that expression of TRIM17 reduced p62’s autophagic degradation (Mandell et al. 2017). Finally, Table VII enlists some of the interactions between autophagy-related proteins and TRIM proteins. Therefore, altogether, results from different groups reveal that TRIM proteins regulate autophagy, however, it remains to clarify the biochemical mechanisms by which each TRIM member functions precisely at a specific event in autophagosome formation and also the significance of its E3 ligase activity in autophagy (Hatakeyama 2017).

Table VII : Interactions between some of the autophagy-related proteins and TRIM proteins. From (Hatakeyama 2017)

<table>
<thead>
<tr>
<th>Autophagy-related proteins</th>
<th>TRIM proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Atg8</td>
<td>TRIM5, 17, 20, 21, 22, 28, 41, 49, 55</td>
</tr>
<tr>
<td></td>
<td>TRIM21, 28</td>
</tr>
<tr>
<td></td>
<td>TRIM5</td>
</tr>
<tr>
<td>GABARAP</td>
<td>TRIM5,16, 17, 20, 22, 49, 55</td>
</tr>
<tr>
<td>p62</td>
<td>TRIM5, 13, 17, 21, 22, 28, 49, 50, 55, 55, 55</td>
</tr>
<tr>
<td>Optineurin</td>
<td>TRIM76</td>
</tr>
<tr>
<td>Others</td>
<td>TRIM5, 6, 17, 20, 21, 22, 40, 49, 61</td>
</tr>
</tbody>
</table>
**Innate Immunity**

Innate immune system provides the first line of defense against pathogen invasion. Interferons, cytokines that induce an anti-viral state through mechanisms involving complex signal cascades mediates the innate immune response to viral infection (Nisole, Stoye, and Saïb 2005). Evidence suggest that TRIM proteins are involved in the induction of these pathways or act as effectors of the anti-viral state (Yap and Stoye 2012). The evidence includes studies demonstrating that upon viral infection, the expression levels of TRIM genes are up regulated in an interferon-dependent manner (Geiss et al. 2002; Barr, Smiley, and Bushman 2008a; Rajsbaum, Stoye, and O’Garra 2008) and studies showing direct interaction of TRIM proteins with different viruses. These TRIM members include TRIM1 (Yap et al. 2004; F. Zhang et al. 2006), TRIM5 (Yap et al. 2004; Stremlau et al. 2004; Hatzioannou et al. 2004), TRIM19 (Everett and Chelbi-Alix 2007), TRIM22 (Barr, Smiley, and Bushman 2008a; Tissot and Mechti 1995), TRIM25 (Michaela U. Gack et al. 2007), TRIM28 (Wolf, Hug, and Goff 2008), TRIM32 (Fridell et al. 1995), and TRIM34 (F. Zhang et al. 2006).

The first TRIM protein that was linked to innate immunity is TRIM5α (Stevenson 2004). Indeed, it was shown that TRIM5α targets several viruses (retroviruses and lentiviruses), to prevent their infection. Conversely, some TRIM proteins are targeted by several viruses for immune escape. For example, Influenza A virus nonstructural protein1 (NS1) interacts with TRIM25 and inhibits the ubiquitination of retinoic acid-inducible gene-I (RIG-I) mediated by TRIM25 resulting in the inhibition of RIG-I-mediated IFN production (Michaela Ulrike Gack et al. 2009). The knockdown of TRIM22 increases the production of HIV-1 particles in the presence of interferon β which suggests that TRIM22 might be a downstream effector of the interferon β response to the HIV-1 infection (Barr, Smiley, and Bushman 2008b).

Hence, TRIM proteins act as critical regulators of innate immunity, however, we need further biochemical analysis and in-vivo analysis using knockout mice models to fully understand the mechanisms by which TRIM proteins orchestrate innate immunity (Hatakeyama 2017).

**Cell cycle regulation and Mitosis**

The eukaryotic cell cycle is defined as the series of events which comprises synthesis of DNA (S-phase), and cell division (M-phase) with intervening gap phases that allows cell growth (G1-phase) and that allows to check the integrity of genomic material (G2-phase). The different checkpoints (G1 and G2) govern the transition through the cell cycle phases and the requirement of different cyclins during different phases of the cell cycle (Figure 31A) (Bai, Li, and Zhang 2017; B. Singh and Wu 2019). Amongst the phases of cell cycle, mitosis is a delicate event where segregation of chromosome into two daughter cells takes place. It is very critical for each daughter cell to receive an exact copy of the genetic material and defects in segregation of the chromosome have been associated with tumorigenesis (Mitelman 1994).

Regulation of phase transitions during the cell cycle by TRIMs: Many TRIM proteins have been associated with cell cycle progression. Figure 31B highlights major and critical TRIM family members which play a role in cell cycle phase transitions and the mitotic progression. For most TRIM
proteins, silencing generally increases the percentage of cells in G0/G1 and reduces cells in the S or G2-M phase. For example, silencing of TRIM8, TRIM14, TRIM27, TRIM28, TRIM29, TRIM52, TRIM59, TRIM66, and TRIM68 resulted in cell cycle arrest (Qi et al. 2016; W. Xu et al. 2016; Benke et al. 2018; Y. Zhang et al. 2018; Aierken et al. 2017; Zhan et al. 2015; Y. Chen et al. 2015; Z. Tan et al. 2017; Venuto et al. 2019; Caratozzolo et al. 2017; Y. Liu et al. 2017). Two different studies have associated TRIM39 with cell cycle. In one study, knockdown of TRIM39 induced G1/S arrest and inhibited proliferation (Liguo Zhang et al. 2012a) whereas in another study, silencing of TRIM39 abrogated G2 checkpoint induced by genotoxic stress which led to increased mitotic entry and, ultimately, apoptosis (Lei Zhang et al. 2012a). Interestingly, unpublished results from our team revealed that the knockdown of TRIM17 resulted in a decrease in cell proliferation and aberrant mitosis with signs of endoreplication and cytokinesis defects.

The targets of TRIM proteins during cell cycle progression include crucial factors such as p53, p21, and/or signalling pathways such as JAK/STAT pathway. For example, ablation of TRIM52 increases the level of activated p53 and in turn of p21 (a cyclin-dependent kinase inhibitor) which is one of the major targets of p53. This results in the inhibition of cyclinE/CDK2 thereby prevents G1/S transition (Karimian, Ahmadi, and Yousefi 2016). In addition, knockdown of TRIM28 raises the expression of p21 significantly and alters p53 slightly (Qi et al. 2016; Benke et al. 2018). On the other hand, TRIM8 physically interacts, stabilizes, and activates p53 protein and this results in the suppression of cell proliferation due to a cell cycle arrest in G1 which is p53 dependent in human osteosarcoma cell lines (Caratozzolo et al. 2017). The association of both TRIM8 and TRIM29 with JAK/STAT signaling pathway may have an effect on cell cycle progression. TRIM8 interacts with and deregulates STAT3 target genes that are involved in cell cycle progression while knockdown of TRIM29 increases the level of JAK2 and STAT3 phosphorylation (W. Xu et al. 2016; Venuto et al. 2019).

Importantly, p53 ubiquitination and stability is controlled by several E3 ubiquitin ligases. p53 is a tumour suppressor protein which protects cells in the times of stress by regulating cell cycle arrest, apoptosis, cellular senescence, DNA repair, and autophagy (Lane 1992; Vogelstein, Lane, and Levine 2000). Indeed, p53 mutation or deficiency is the contributing factor in several human tumours. Although MDM2 had been identified as a primary determinant of p53 ubiquitination and stability, it is clearly not the only p53 -directed ligase. This was evident when the mutation in MDM2 did not prevent p53 ubiquitination and degradation. Apart from MDM2, TRIM24 and TRIM39 have been identified as E3 ubiquitin ligases of p53 where they promote degradation of p53 and regulate its stability (Liguo Zhang et al. 2012a). In a separate study, it was also shown that TRIM39 stabilizes p21 by blocking its ubiquitination and proteasomal degradation by CRL4Ch2 E3 ubiquitin ligase. This stabilization of p21 by TRIM39 regulates cell cycle progression and the balance between cytokinesis and apoptosis after DNA damage (Lei Zhang et al. 2012a).

Altogether, different stages of cell cycle progression and mitosis are regulated by TRIM proteins. Owing to their involvement during the cell cycle, the deregulation of expression of TRIMs has been directly linked to an increase in percentage of cells in specific cell cycle phases (G1-S-G2 or M), modulation of cell proliferation and cell death resistance (Venuto and Merla 2019).
Regulation of kinetochore by TRIMs: Kinetochore is a large protein complex central for the alignment and segregation of chromosomes from the onset of mitosis which ensures the connection of sister chromatids to the opposite spindle poles. The timed segregation during mitosis is governed by a large number of protein components of the kinetochore that helps in the prevention of incorrect attachment of microtubules (Nagpal and Fukagawa 2016). Among the TRIM proteins, TRIM17, TRIM36 and TRIM69 play an important role in this process by interaction with proteins from the kinetochore (Endo et al. 2012a; Sinnott et al. 2014; Miyajima et al. 2009). Therefore, TRIM proteins interact and regulate the proteasome-dependent degradation of several kinetochore proteins. Altogether, it is evident that TRIM proteins may act as either tumour suppressor or as oncogenes by controlling proliferation of cells and mitosis (Venuto and Merla 2019).

### 3.4 Pathological roles of TRIM proteins

The alteration of TRIM proteins results in various human diseases such as neurodegenerative diseases including Alzheimer’s disease (AD), Parkinson disease (PD), multiple sclerosis (MS), various cancers such as neuroblastoma, melanoma, breast cancer, and developmental disorders such as Opitz Syndrome (OS), Williams-Beuren syndrome (WBS), etc. Several TRIM proteins have been identified which contribute to the development of these diseases, however, the exact mechanisms by which TRIM proteins contribute to the pathogenicity remains to be investigated for most of the diseases.

#### 3.4.1 TRIM proteins in neurodegenerative diseases

The biological roles of many TRIM proteins are yet to be discovered, however, given their significance in various cellular processes particularly in apoptosis, studies from various groups...
highlight their involvement in neurodegenerative diseases. The TRIM proteins implicated in few neurodegenerative diseases such as MS, AD, PD are described below.

Table VIII Role of different TRIMs in the regulation of kinetochore. From (Venuto and Merla 2019)

<table>
<thead>
<tr>
<th>TRIM member</th>
<th>Function and effects</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. TRIM17</td>
<td>-Interacts with kinetochore protein, ZWINT and results in its degradation.</td>
<td>Via ubiquitination of ZWINT</td>
<td>(Endo et al. 2012a)</td>
</tr>
<tr>
<td></td>
<td>-The knockdown of ZWINT causes chromosome bridge phenotype, results in abrogation of mitotic arrest and triggers cell death</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-Overexpression of TRIM36 prevents recruitment of CENP-H to centromeres thereby decelerating the cell cycle and attenuating cell growth.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. TRIM69</td>
<td>-essential for proper attachment of microtubules to kinetochores and for mitotic fidelity.</td>
<td>Via its E3 ligase activity, however, needs further investigation</td>
<td>(Sinnott et al. 2014)</td>
</tr>
<tr>
<td></td>
<td>-Its depletion increases occupancy of BUBR1 at the kinetochores. Therefore, it acts as a regulator of mitotic spindle assembly.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Multiple sclerosis

Multiple sclerosis (MS) is a disorder of the central nervous system defined by multiple inflammation and demyelination in the brain and the spinal cord (Watanabe and Hatakeyama 2017). Using genome-wide association study, single nucleotide polymorphisms (SNPs) in TRIM10, TRIM15, TRIM26, TRIM39, and TRIM40 genes were linked to susceptibility to MS (Baranzini et al. 2009).

Individuals with 7 SNPs identified in the first intron of TRIM5 gene in human chromosome 11 have been associated with less predisposition towards MS. As discussed earlier, TRIM5 encodes a ubiquitin ligase that is involved in the restriction of replication of many retroviruses. The association of TRIM5 with MS might thus suggest the involvement of retroviruses in the etiology of MS (Nexo et al. 2011). On the other hand, data suggested that MS patients had higher mutations in TRIM20/Pyrin gene compared to healthy individuals (Unal et al. 2010). Altogether, TRIM family plays an important role in the pathogenesis of MS.

Alzheimer’s Disease

Alzheimer’s disease (AD) is a progressive neurodegenerative disease which is caused by both genetic and environmental factors. Several studies confirm that the central pathway to the disease is the generation of Aβ amyloid from the amyloid precursor protein (APP) (Masters and Beyreuther 1998).
Different studies have linked TRIM proteins to AD. Some of the TRIM proteins involved in the pathogenesis of AD include TRIM2, TRIM11, TRIM20, TRIM21, TRIM32, and TRIM37 (Tocchini and Ciosk 2015; Niikura et al. 2003; Arra et al. 2007; Yokota et al. 2006; McEwan et al. 2017).

TRIM2 is known to be predominantly expressed and function in brain. TRIM2 acts as an E3 ubiquitin ligase and degrades NeuroFilament Light subunit (NF-L) thus preventing neurodegeneration. It has been shown that downregulation of two microRNAs (miR), miR-9 and miR-181, led to an increase in the levels of TRIM2 and this increase has been linked to the onset of AD (Tocchini and Ciosk 2015). TRIM11 contributes to AD by binding humanin (a neuroprotective peptide that suppresses AD-related neurotoxicity) and degrading it (Niikura et al. 2003). Mutation in TRIM20/Pyrin leads to an increase in IL-1β expression which is closely associated with accumulation of β-amyloid plaques and neurofibrillary tangles and this triggers the pathogenesis of AD (Griffin and Mrak 2002; Licastro et al. 2000). TRIM32 and TRIM37 show increased expression in AD occipital lobes (an area with less pathological changes at early stages of AD) links these TRIM members to AD (Yokota et al. 2006), whereas, reduced expression of these TRIM proteins leads to a decrease of neuronal survival.

The role of different TRIM proteins in AD needs further investigation as the mechanisms involved in its pathogenesis are mostly unknown. This research in AD might therefore pave way for the development of therapeutic strategies targeting TRIM proteins.

Parkinson Disease

Parkinson disease (PD) is the second most common neurodegenerative disorder and the motor symptoms of PD result mainly from the neurodegeneration of the dopaminergic neurons of the substantia nigra. SNCA which encodes α-synuclein, an abundant presynaptic protein, was the first gene mutated in familial PD (Corti, Lesage, and Brice 2011; Dehay et al. 2015). Indeed, several studies suggest that an increase in the WT-α-synuclein is enough to cause both the inherited and sporadic forms of PD (Devine et al. 2011; Stefanis 2012).

Alterations in TRIM9 protein were found in neurodegenerative disorders affecting the cerebral cortex and hippocampus. Therefore, when immunohistochemistry of TRIM9 was applied to brain sections from patients with PD, the immunoreactivity in the substantia nigra (a basal ganglia structure located in the midbrain) was diminished in PD. Interestingly, serial sections stained with anti-TRIM9 and anti-α-synuclein antibodies revealed that approximately 80-90% of cortical and brain stem-type LBs were positive for TRIM9. Therefore, the presence of TRIM9 in the LB found in PD suggests that it might participate in the pathogenesis of PD (V. V. Nenasheva et al. 2017).

Lassot et al., identified that ZSCAN21 stimulated SNCA transcription in neuronal cells and that TRIM41 is an E3 ligase of ZSCAN21. In contrast, TRIM17 decreased the TRIM41-mediated degradation of ZSCAN21. Interestingly, using a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) based animal model of PD based on the injection of the neurotoxin MPTP, it was shown that the mRNA levels of TRIM17, ZSCAN21, and SNCA were simultaneously increased in the midbrains of mice following MPTP treatment. Importantly, rare genetic variants in ZSCAN21,
TRIM17 and TRIM41 genes occur in patients with familial forms of PD. Expression of ZSCAN21 and TRIM41 gene variants resulted in the stabilization of the ZSCAN21 protein. This suggests that deregulation of ZSCAN21 by TRIM17 and TRIM41 might be involved in the pathogenesis of PD (Iréna Lassot et al. 2018).

Cancer

TRIM protein family members such as TRIM8, TRIM16, PML/TRIM19, TRIM24, TRIM25, TRIM27, TRIM33, etc. are involved in the regulation of carcinogenesis (Cambiaghi et al. 2012a; Meroni 2012b). Of these, four TRIM family members namely, TRIM19, TRIM24, TRIM27, and TRIM33 acquire oncogenic activity upon chromosomal translocations. TRIM proteins impact different cancers through multiple mechanisms and few of them would be described in the following paragraphs.

TRIM proteins in chromosomal translocations

PML (ProMyelocytic Leukaemia)/TRIM19: PML was identified by cloning the breakpoint sites of the t (15:17) chromosome translocation associated with Acute Promyelocytic Leukaemia (APL) (Goddard et al. 1991; de Thé et al. 1991). It becomes oncogenic when its RBCC motif recombines with the product of the retinoic acid receptor (RARα) gene. RARα is a member of the nuclear hormone receptor superfamily of transcription factors and its fusion with PML results in a chimera protein PML-RARα. In APL, owing to the increased affinity for transcriptional corepressors and HDACs, PML-RARα acts as a potent dominant negative inhibitor of the WT-RARα. This interaction leads to transcriptional repression and consequent block of differentiation (Cambiaghi et al. 2012a).

In contrast, tumour suppressor function of PML can be evidenced from knockout studies in mice. When PML was knocked out in mice, they developed normally and did not get spontaneous cancers but instead were more sensitive to tumour promoting agents. This suggested that PML could antagonize the initiation, promotion and progression of tumours (S. Zhong et al. 1999b).

TRIM27/RFP: TRIM27 is another TRIM member which becomes oncogenic when its RBCC motif fuses with another protein such as RET proto-oncogene. This translocation results from the DNA rearrangement that occurs during transfection of the NIH 3T3 cells with human T-cell lymphoma DNA. Consequently, the resulting transforming protein TRIM27/RET displays the fusion of the tripartite motif of TRIM27 with the C-terminal tyrosine-kinase domain of the RET protein. Interestingly, a correlation between TRIM27 and ERBB2 protein expression as well as with the amplification of ERBB2 gene in breast cancer has been observed which implicates TRIM27 in the pathophysiology of cancer (Tezel et al. 2009).

Further, a study provided evidence for the physical and functional interactions between the retinoblastoma tumour suppressor protein (Rb) and TRIM27. The Rb activating function might be inhibited by TRIM27 due to the induction of the stability of EID-1 (E1A-like inhibitor of
differentiation), a histone acetyl-transferase (HAT) inhibitor by TRIM27 (Krützfeldt et al. 2005). All these data suggest that TRIM27 may act as an oncogene depending on the context (Cambiaghi et al. 2012b).

**TRIM proteins as E3-Ub ligases in oncogenesis**

TRIM25/Oestrogen-responsive Finger Protein (EFP): TRIM25/EFP is highly expressed in breast cancer and apparently, the ovarian hormone oestrogen has been associated with the stimulation of breast tumor growth. Two ligand-dependent transcription factors ERα and ERβ, which are highly upregulated in breast cancer, are required for a direct effect of oestrogen on oestrogen responsive tissues. Interestingly, TRIM25 is a downstream target gene of ERα and has an important role in mediating oestrogen action in breast cancer (K. Ikeda et al. 2000). Functionally, TRIM25 acts as an E3 ubiquitin-ligase and directly degrades 14-3-3-σ (a cell-cycle regulatory protein) in a ubiquitin-dependent manner. This degradation of 14-3-3-σ results in the dissociation of the protein with cyclin-Cdk complexes which leads to cell-cycle progression and tumour growth. Therefore, by degrading the cell cycle regulator 14-3-3-σ, EFP might contribute to the deregulated proliferation of breast cancer cells (Urano et al. 2002).

TRIM32: TRIM32 was originally identified in the mouse clonal epidermal model of carcinogenesis. TRIM32 is elevated in a fraction of human head and neck squamous cell carcinoma (HNSCC). TRIM32 is an E3 ubiquitin-ligase of PIASy (protein inhibitors of activated STATs), a SUMO-E3 ligase involved in the control of apoptosis, senescence, and NFκB activation. In response to UVB and TNFβ-induced stress, Piasy promotes keratinocyte apoptosis via the inhibition of NFκB survival function. The importance of this interaction in the development of human HNSCC remains to be investigated since this mechanism might have important clinical implications (Albor et al. 2006).

In contrast, TRIM32 can also act as a tumour suppressor. In mammalian cells, TRIM32 interacts and ubiquitinates XIAP (X-linked inhibitor of apoptosis), therefore inducing tumour necrosis factor (TNF)-mediated apoptosis. Hence, degradation of XIAP prevents its inhibition of pro-apoptotic caspases. These findings, suggest that TRIM32 can be exploited as a therapeutic target in various cancers (Ryu et al. 2011).

### 3.5 TRIM17

Trim17 has been the subject of interest in our laboratory for 15 years. Indeed, based on a microarray screen, Solange Desagher identified Trim17 as one of the genes most highly upregulated during serum and KCl deprivation-induced apoptosis in our cerebellar granule neurons model (CGN) (Desagher et al. 2005; Irina Lassot et al. 2010a). Many studies done in our group highlight the significance of Trim17 (Irina Lassot et al. 2010a, 17; Magier et al. 2013a; Mojsa et al. 2015a; Lionnard et al. 2019; Iréna Lassot et al. 2018). During my PhD, I focussed on Trim17 and its partners
Trim39 and NFATc3 (described in next chapter). In this chapter, therefore, I would be describing Trim17 and Trim39 in particular.

**Expression of Trim17**

Trim17 (Tripartite motif-containing 17) also named RNF16 (Ring Finger protein 16) or terf (testis RING finger protein) is a member of the Class IV RBCC subfamily (Figure 26). It was identified and cloned for the first time in rat testis (Sumito Ogawa et al. 1998). The chromosomal mapping revealed that human *TRIM17* is located at 1q42, mouse *TRIM17* at 11B1.2-B1.3 and rat *TRIM17* at 10q22 (S Ogawa et al. 2000). On the other hand, the sequence alignment data reveals that rat *TRIM17* is 74% identical to human *TRIM17* whereas 95% identical to the murine *TRIM17*. Using northern blot analysis and real time RT-PCR, it was demonstrated that Trim17 was predominantly expressed in human and rat testis (Sumito Ogawa et al. 1998). Apart from testis, it was found to be significantly expressed in the brain, spleen and thymus (Urano et al. 2009) and to a lesser extent in liver, and kidney (Urano et al. 2009). Public databases also report an important expression in different parts of the brain (Figure 32). The molecular weight of Trim17 protein was calculated to be around 54.9 KDa in rat and 54.3KDa in humans (Sumito Ogawa et al. 1998). Incidentally, murine and human orthologs have two isoforms which varies in their CTD, however, the truncated form of the protein lacks the PRYSPRY domain.

**Structure of TRIM17**

The structure of TRIM17 has not been determined yet, however, the predicted homodimeric 3D structure encompassing CC and the PRY-SPRY domain of human TRIM17 (Q9Y577) is available on the Swiss-Model Repository database (Figure 33). The 3D structure was generated based on the
sequence homology of the CC and PRY-SPRY domain of TRIM20 with whom TRIM17 shares 26.27% sequence identity.

**Figure 33:** Predicted homodimeric 3D structure of CC and PRY-SPRY domain of hTRIM17 (Q9Y577) based on TRIM20 (From Swiss Model Repository database)

### 3.5.3 Partners and substrates of TRIM17

The GST-pulldown assay using GST-Trim17 produced in *E. coli* and a lysate from apoptotic CGN revealed 27 candidates (unpublished data Irina Lassot). The putative interactors included proteins involved in RNA metabolism (Ybx1, RpIp0, Rps4x, Eif2b1, Srp RBI, Elavl2, Elavl4, Ddx17), cytoskeleton-related proteins (Dst, Myh10, Tpm4, Myo5a, Map1a), signalling pathways (Ywhag, Pgam5, Ywhaq, Ddah2, Ywhae), vesicular trafficking (Vapa, Snap91), transcription (Morf412, Gtf3c1), and proteins whose function has not been identified yet (Sle25a11, Psm7, Spin1, Ankrd17, Phb) (Figure 34). In addition, using Y2H screen (Hybrigenics), our group identified 19 putative partners which included two transcription factors: NFATc3 (Nuclear factor of activated T cells) (Mojsa et al. 2015a) and, ZSCAN21 (Irena Lassot et al. 2018), and one TRIM protein: TRIM41 (Irena Lassot et al. 2018). Apart from TRIM41, Trim17 can interact with other TRIM proteins including TRIM28 (Lionnard et al. 2019), TRIM39 (Woodsmith, Jenn, and Sanderson 2012), and TRIM44 (Urano et al. 2009). Other partners that have been identified include NFATc4 (Mojsa et al. 2015a), BCL2A1 (Lionnard et al. 2019), Mci-1 (Magiera et al. 2013a), p62 (Mandell et al. 2017), and ZWINT (Endo et al. 2012a) to name a few. Finally, according to an extensive study on the functional interactions between E2s and E3s, TRIM17 can directly bind to the following E2s: UBE2D1, D2, and D3, which is in line with the data generated in our group.

Therefore, given that the different interactors of TRIM17 are crucial regulators of diverse cellular processes, TRIM17 has a critical role in many different cellular processes such as apoptosis, signalling, autophagy, cell-cycle regulation, etc. Few of the known substrates and partners of Trim17 would be described below.
Substrates of Trim17

Myeloid cell leukemia-1 (Mcl-1) is an anti-apoptotic protein of the Bcl-2 family and is characterized by a short half-life. It is expressed in CGNs in-vivo, and its expression has been associated with neuroprotection in the hippocampus. It was demonstrated that Trim17 could mediate the ubiquitination and proteasomal degradation of Mcl-1 both in CGNs and in vitro. Indeed, in-vitro studies further confirmed that Mcl-1 was directly ubiquitinated by Trim17 (Magiera et al. 2013a). Apart from Mcl-1, it was also shown that TRIM17 decreased the protein levels of ZWINT, a kinetochore protein, although, the mechanism by which TRIM17 induces the degradation of ZWINT was not examined (Endo et al. 2012b).

Partners of Trim17

My group also demonstrated that Trim17 bound to both NFATc3 and NFATc4, and inhibited their nuclear translocation (Mojsa et al. 2015a). BCL2A1 is an anti-apoptotic protein of the BCL-2 family. It was shown by our group that Trim17 could interact with both BCL2A1 and TRIM28 (an E3 ligase of BCL2A1). Indeed, Trim17 stabilized BCL2A1 by blocking TRIM28 from binding and ubiquitinating BCL2A1 (Lionnard et al. 2019). One possibility is that TRIM17 forms an inactive hetero-oligomer with TRIM28 which cannot bind BCL2A1. Alternatively, there might be competition between TRIM17 and TRIM28 to bind BCL2A1, however, these two possibilities are not mutually exclusive.

Lassot et al., 2018 showed that TRIM17 could interact with the transcription factor ZSCAN21 and its E3 ubiquitin-ligase TRIM41. In a similar mechanism as above, TRIM17 inhibits the interaction of TRIM41 and ZSCAN21 thereby inhibiting the ubiquitination of ZSCAN21 mediated by TRIM41. Two possible mechanisms for the inhibitory effect of TRIM17 include, first, a direct inhibition of the

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**Figure 34: GST-pulldown of TRIM17 (Irina Lassot: unpublished data)**
E3 ligase activity of TRIM41 by TRIM17. Indeed, TRIM17 inhibited the auto-ubiquitination of TRIM41 possibly by forming inactive hetero-oligomers. Second, disruption of the interaction of ZSCAN21 and TRIM41 by TRIM17. These two mechanisms are not mutually exclusive, as formation of hetero-dimers between TRIM17-TRIM41 may prevent binding of TRIM41 to ZSCAN21 (Iréná Lassot et al. 2018).

Interestingly, it was also shown by TRIM17 interacts with TRIM44 (a RING-less TRIM member) and this interaction led to the stabilization of TRIM17 at the protein level. Furthermore, TRIM44 reduced the ubiquitination of TRIM17 (Urano et al. 2009). Finally, TRIM17 is also known to stabilize p62 and to prevent its autophagic degradation (Mandell et al. 2017).

3.5.4 Molecular and cellular functions of Trim17

**Molecular function of Trim17**

Trim17 as an E3 ubiquitin ligase: Urano et al. 2009, and Lassot et al., 2010 demonstrated that Trim17 is an E3 ubiquitin-ligase as both, the human and mouse TRIM17, have been shown to auto-ubiquitinate in-vitro in the presence of E2’s: Ube2e1, Ube2d2, and Ube2d3. Indeed, the first substrate identified of Trim17 was Mcl-1 (Magiera et al. 2013b). Interestingly, TRIM17 induced the degradation of a kinetochore protein, ZWINT (Endo et al. 2012a).

**Cellular functions of TRIM17**

Apoptosis: My group showed that Trim17 initiates neuronal apoptosis. Using overexpression and silencing of Trim17, it was shown that Trim17 was necessary and sufficient to induce neuronal apoptosis both in CGN and sympathetic neurons in a Bax-dependent manner (Irina Lassot et al. 2010a). Importantly, the pro-apoptotic effect was dependent on its RING domain, so potentially on its E3 activity. Indeed, inactive mutants of Trim17 (deletion: ΔRING or substitution mutant: C16A) exerted a dominant-negative function when they were overexpressed in CGN and sympathetic neurons as it resulted in their protection from apoptosis. Therefore, our group identified that Trim17 was a crucial E3 ubiquitin-ligase that initiated neuronal apoptosis (Irina Lassot et al. 2010a).

Importantly, as Trim17 was identified as the E3 ubiquitin-ligase of Mcl-1, the pro-apoptotic effect of Trim17 in neurons might be involved, at least in part, in the ubiquitin-mediated degradation of the anti-apoptotic Mcl-1 (Magiera et al. 2013a).

Furthermore, studies suggest that NFATc4 sustains survival in different types of neurons notably by inducing the transcription of survival factors (Quadrato et al. 2012). Mojsa et al., showed that the CGNs were protected from apoptosis after the transfection of NFATc4. Therefore, the inhibition of the prosurvival factor NFATc4 by Trim17 might partially mediate the proapoptotic effect of Trim17 in CGNs (Mojsa et al. 2015a).
Lionnard et al., showed that Trim17 was induced following treatment of melanoma cells with PLX4720, an anti-cancer drug. Hence, TRIM17 might participate in chemoresistance, but also in tumorigenesis in cells undergoing chronic stress, by preventing TRIM28-mediated degradation of BCL2A1 and by increasing the protein levels of this anti-apoptotic protein (Lionnard et al. 2019).

**Cell cycle progression:** *ZWINT* is a kinetochore protein which is required for the mitotic spindle checkpoint. A study showed that stable expression of ZWINT in MCF breast cancer cells increased their cellular proliferation while TRIM17, in contrast, reduced their proliferation by inducing the degradation of ZWINT (Endo et al. 2012a). Additionally, our team observed that the knockdown of **TRIM17** resulted in a decrease in cell proliferation and aberrant mitosis with signs of endoreplication and cytokinesis defects (unpublished data). Therefore, these results suggest that TRIM17 can exert opposite effects on cell cycle regulation which might be substrate and context dependent.

**Autophagy:** As mentioned before, Mandell et al., observed that the amount of *p62* was reduced after the knockdown of TRIM17 suggesting that TRIM17 inhibited the autophagic degradation of p62. Indeed, TRIM17 expression also prevented degradation of p62 in cells which were treated with pp242 (drug that induces autophagy through mTOR inhibition). In addition to p62, TRIM17 also interfered with the autophagic degradation of another target of selective autophagy, **IFT20** (Mandell et al. 2017). Furthermore, TRIM17 could interact and co-localize with **TRIM5α** and **TRIM22** (Imam et al. 2016) protecting these TRIM proteins from proteolysis. Taken together, these results suggest that TRIM17 is an inhibitor of selective autophagy (Mandell et al. 2017).

**Transcriptional Regulation:** TRIM17 regulates the transcription by acting on transcription factors NFATc3, NFATc4 and ZSCAN21. Mojsa et al., observed that the binding of Trim17 to **NFATc3** impacted its transcriptional activity as a decrease of NFATc3-mediated luciferase expression was observed after co-transfection of WT-Trim17. Moreover, the overexpression of Trim17 decreased the mRNA levels of BDNF, a target gene of NFATc3. Interestingly, Trim17 also decreased the reporter gene activity mediated by **NFATc4** (Mojsa et al. 2015a).

The involvement of TRIM17 in regulating the stability of **ZSCAN21** in the context of PD has been described above. Indeed, Lassot et al., observed that silencing of endogenous ZSCAN21 or **TRIM17** resulted in a significant decrease in the mRNA level of **SNCA**. These results suggested that TRIM17 regulated the **SNCA** expression (Iréna Lassot et al. 2018).

### 3.5.5 Trim17 and disease

High through put screenings linked the mutations in **TRIM17** gene with various disorders. For example, the missense mutation in **TRIM17** gene, V132L and G326V, were found to be associated with multiple myeloma (Chapman et al. 2011), and recessive cognitive disorders (Najmabadi et al. 2011), respectively. Further, TRIM17 has been linked to autism owing to an insertion in its
PRYSPRY domain (Iossifov et al. 2012). In addition to this, a candidate-gene testing for orphan limb-girdle muscular dystrophies (LGMD) identified mutations in the TRIM17 gene in LGMD patients, however, the correlation of these mutations with LGMD phenotype remains to be investigated (Aurino et al. 2008). TRIM17 regulates the transcription of the SNCA gene. Five rare exonic variants in TRIM17 were identified in patients with autosomal dominant PD compared to healthy controls. Notably, four of these TRIM17 variants were predicted to be damaging using in silico prediction (Iréna Lassot et al. 2018). Finally, Lionnard et al, 2019 showed that depletion of TRIM17 restored the sensitivity of melanoma cancer cells (SK-MEL-28 cells) to PLX4720-induced apoptosis. Therefore, TRIM17 could be targeted to promote BCL2A1 degradation in order to restore sensitivity of BCL2A1-dependent cancer cells towards chemotherapeutics (Lionnard et al. 2019). Altogether, Trim17 can be involved in the regulation of many pathologies.

3.6 TRIM39

In a Y2H screen, three studies had identified that TRIM17 and TRIM39 physically interact with each other (Rual et al. 2005; Woodsmith, Jenn, and Sanderson 2012; Rolland et al. 2014). This section will be dedicated towards the details of its expression, structural aspects, functions, its substrates and partners.

3.6.1 Expression of TRIM39

![Gene expression for TRIM39 (ENS0000020599.14)](image)

TRIM39 (Tripartite motif-containing 39), also named RNF23 (RING Finger Protein23) or TFP (Testis abundant finger protein) is a part of Class IV RBCC subfamily, like TRIM17 (Figure 26). It was isolated from human and mouse testis cDNA libraries. The open reading frames of human and
mouse forms of TRIM39 comprises of 519 and 489 amino acids, respectively and encodes for RING, B-Box, Coiled Coil and B30.2/PRY-SPRY domains. Interestingly, the human and mouse orthologues of TRIM39 share 98% identity. It should be noted that compared to the human TRIM39, the mouse form lacks 30 amino acids (269-298 aa) due to alternative splicing. The molecular weight of human TRIM39 is predicted to be around 57KDa whereas that of mouse form at 53.79 KDa. The human TRIM39 lies within the major histocompatibility complex class I region on chromosome 6p21.3-p22.1 (Orimo et al. 2000). TRIM39 mRNA is ubiquitously expressed in various organs, however, it is highly expressed in the testis, brain, heart, kidney, liver, skeletal muscle, and spleen (S. S. Lee et al. 2009a). Public databases (Figure 35) also confirm that TRIM39 is highly expressed in testis and in the brain particularly cerebellum.

![Figure 36: 3D Solution structure of the RING domain of human TRIM39 (Source: 2ECJ, Sehnal et al., 2018, RCSB PDB)](image)

![Figure 37: 3D Solution structure of the B-Box domain of human TRIM39 (Source: 2ECJ, Sehnal et al., 2018, RCSB PDB)](image)
3.6.2 Structure of TRIM39

The solution structure of the RING domain (2ECJ) and the B-Box domains of the human TRIM39 (2DID) was determined using solution Nuclear Magnetic Resonance (NMR) technique. This powerful biophysical tool is used to ascertain atomic resolution details of a protein (Puthenveetil and Vinogradova 2019). The human TRIM39 is a 518 aa protein and Figure 36 and Figure 37 highlights the amino acids encompassing the RING domain and the B-Box domain. The solution structure of the coiled-coil and the PRY-SPRY domain is yet to be determined.

3.6.3 Partners and substrates of TRIM39

TRIM39 is known to interact with many proteins. Some of its known partners include Modulator of Apoptosis-1 (MOAP-1) (S. S. Lee et al. 2009a, 1; N.-J. Huang et al. 2012a), several UbE2 enzymes (Woodsmith, Jenn, and Sanderson 2012), p21 (Lei Zhang et al. 2012), Cactin (Suzuki et al. 2016), several TRIM protein family members including TRIM17 (Woodsmith, Jenn, and Sanderson 2012) and, zinc-finger proteins. p53, on the other hand, was identified as a genuine substrate of TRIM39 (Liguo Zhang et al. 2012a).

The interaction network of TRIM39 is shown in the Figure 38 (Chatr-Aryamontri et al. 2013). The interactions of TRIM39 with its partners and substrate were confirmed using a Y2H screen (TRIM17, MOAP-1 and Cactin) and using biochemical techniques such as co-immunoprecipitation (TRIM17, MOAP-1, Cactin, p21, p53).

Substrates of TRIM39: It was demonstrated that Trim39 could interact and regulate the stability of p53. The half-life of p53 was significantly increased upon knockdown of Trim39. A direct interaction and in-vitro ubiquitination of p53 by Trim39 further supported the ability of Trim39 to directly regulate p53 (Liguo Zhang et al. 2012a).


p21 is a cyclin-dependent kinase inhibitor (CKI) that is capable of inhibiting all cyclin/CDK complexes and links DNA damage to cell cycle arrest. TRIM39 interacts with p21, maintains the steady state levels of p21 and protects p21 from ubiquitin-mediated degradation by competing with its E3 ubiquitin-ligase CRL4Cd2 for p21 interaction (Lei Zhang et al. 2012a).

Cactus-interactor (Cactin), also known as renal carcinoma antigen NY-REN-24, is a protein which inhibits NFkB and TLR-mediated transcriptions. TRIM39 interacts and stabilizes Cactin protein, however, the exact mechanism by which TRIM39 stabilizes Cactin is not known yet (Suzuki et al. 2016).
Molecular and cellular functions of TRIM39

As other TRIM members, TRIM39 has an E3 ubiquitin ligase activity which is conferred by its RING domain.

Molecular function of Trim39

Trim39 is an E3 ubiquitin-ligase: The primary sequence analysis of human Trim39 revealed that it is a close ortholog of an E3 ligase from *Xenopus laevis* known as Xnf7. As Xnf7 regulates mitotic exit by inhibiting Anaphase Promoting Complex (APC/C- a E3 ubiquitin-ligase) (Casaletto et al. 2005), it was speculated that Trim39 could be an E3 ligase as well. Indeed, the *in-vitro* studies using recombinant E1, E2 (UbcH5a), ubiquitin and Trim39 as the only source of E3 activity demonstrated that Trim39 could undergo auto-ubiquitination (N.-J. Huang et al. 2012a).

Trim39 was identified as an E3 ligase for p53 which directly regulates its stability. Results from the study indeed show that Trim39 contributes more significantly than MDM2 to cell growth and the stability of p53. By modulating p53, TRIM39 controls key cellular processes such as apoptosis and cell cycle progression (Liguo Zhang et al. 2012a).
Cellular functions of Trim39

Apoptosis: Etoposide is one of the apoptotic stimuli that can induce the upregulation of endogenous MOAP-1. The knockdown of TRIM39 resulted in a significant decrease in etoposide-induced apoptosis whereas the overexpression of TRIM39 enhanced apoptosis. Further, the data of overexpression and knockdown of TRIM39, suggests that elevated levels of MOAP-1 caused by TRIM39 could result in enhanced Bax-mediated cytochrome c release and hence increased sensitivity of apoptotic stimuli (S. S. Lee et al. 2009a). Altogether, the data from this study suggested that TRIM39 affects the cellular levels of MOAP-1 and acts as a regulator of Bax-dependent apoptosis by increasing the cellular levels of MOAP-1 (S. S. Lee et al. 2009a).

As mentioned above, Trim39 can ubiquitinate p53. Numerous pharmacological approaches have been taken to upregulate or stabilize p53 protein in tumours with WT p53. Nutlin-3a and MI-219, have shown to disrupt the interactions of p53 and its ubiquitin-ligase MDM2 (Vassilev et al. 2004; Shangary et al. 2008; Vassilev 2007) but not that of p53 and Trim39. It was shown that a combination of knockdown of Trim39 and treatment of nutlin-3a triggered apoptosis in cells which were insensitive to nutlin-3a in some cell lines. In this case, however, Trim39 can be considered as an anti-apoptotic protein as its loss promotes the stabilization of p53 and cell death (Liguo Zhang et al. 2012a).

Finally, as DNA damage induces p21, Zhang et al. assessed the effect of TRIM39 depletion on apoptosis induced by doxorubicin or etoposide (DNA damaging agents). Upon Trim39 depletion, cells became more apoptotic by 24h of genotoxic treatment compared to control. Silencing p21 also exerted similar effects on DNA damage induced apoptosis, however, when p21 was re-introduced, it protected the cells from apoptosis induced by doxorubicin or etoposide. These data suggest that the ablation of TRIM39 sensitizes cells to DNA damage induced apoptosis via blunting the accumulation of p21. Therefore, Trim39 might be a crucial regulator in triggering cytostasis (Lei Zhang et al. 2012a).

Therefore, it can be stated that depending on the context, TRIM39 can be either pro-apoptotic or anti-apoptotic.

Cell cycle regulation: TRIM39 regulates the progression of cell cycle and DNA damage responses by stabilizing p21, a known target of p53. Depletion of TRIM39 led to destabilization of p21 and an increase in G1/S transition. In contrast, when TRIM39 was overexpressed, the cells were arrested in the G1 phase (Lei Zhang et al. 2012a). These data indicated p21 as a critical downstream effector of TRIM39 in mediating G1/S transition (Lei Zhang et al. 2012a). Further, it was demonstrated that the G1/S arrest resulting from Trim39 depletion in RPE cells was exerted via p53. Altogether, the data suggested that accumulation of p53 and its target, p21, can be induced by Trim39 depletion which results in a failure to traverse the G1/S transition (Liguo Zhang et al. 2012a).
Regulation of NFκB signalling pathway: NFκB is a critical regulator of cell survival, immunity, inflammation, carcinogenesis, and organogenesis. It was demonstrated that TRIM39 negatively regulates NFκB signaling through the stabilization of Cactin (Suzuki et al. 2016). Indeed, the overexpression of TRIM39 suppressed NFκB-mediated transcriptional activity whereas silencing of TRIM39 promoted its transcriptional activity.

3.6.5 TRIM39 in disease

Behcet’s disease (BD) is a chronic systemic inflammatory disease which is characterized by recurrent oral and genital ulcers, skin lesions, and uveitis. One of the SNPs associated with BD was found to be located on the exon 9 of TRIM39 in the Japanese population. Thus, TRIM39 may be involved in developing BD (Kurata et al. 2010). Cutaneous lupus erythematosus (CLE) is a chronic autoimmune disease of the skin. Single Nucleotide Polymorphisms (SNPs) associated with the disease are located within the MHC region of chromosome 6. Interestingly, TRIM39-RPP21 locus represents naturally occurring read-through transcription between the neighbouring TRIM39 and RPP21 (Ribonuclease P/MRP 21kDa subunit) genes on chromosome 6. Recent data showed that TRIM39R, the TRIM39/RPP21 read through transcript mediates interferon response. So, these genes might interfere with the regulation of apoptosis and interferon response, which are known to play a role in CLE and provide a possible explanation on the deregulated interferon and apoptosis response in CLE (Kunz et al. 2015).
Although initially identified as an inducible nuclear factor that could bind the interleukin-2 (IL-2) promoter in activated T cells, the expression of NFAT (Nuclear Factor of Activated T-cells) is not limited to T cells. Since their discovery more than 25 years ago, NFAT proteins have been found to play key roles in the development and function of both immune and non-immune cells. NFAT have complex mechanisms of regulation and their ability to integrate calcium signalling with other signalling pathways provides an explanation for the functional versatility of NFAT proteins (Macian 2005). NFAT play an important role in many developmental programmes including T-cell differentiation, osteoclast differentiation, cardiac valve development, and neuronal and vascular patterning. Further, NFAT play an important role in various cellular functions such as apoptosis, transcriptional regulation, cell-cycle regulation to name a few and are therefore implicated in many diseases such as auto-immune diseases, cancer, neurodegenerative diseases, etc. (Feske, Rao, and Hogan 2007). In this chapter, I would describe NFAT family in general, their regulation, physiological roles, molecular and cellular functions, and how they are implicated in diseases. Further, I would describe NFATc3 and its role in neuronal apoptosis later, as the topic of interest of my PhD.

Figure 39: Schematic representation of human calcium-regulated NFAT genes and their isoforms. From (Mognol et al. 2016a)
4.1 NFAT family

NFAT denotes a family of transcription factors which is composed of five members. NFAT members are encoded by distinct genes and alternative splicing results in different isoforms of each gene (Figure 39) (Mognol et al. 2016a). Four genes are calcium (Ca\(^{2+}\))-regulated transcription factors, namely, NFATc1 to NFATc4. The fifth gene, NFATc5 was identified as the tonicity-responsive enhancer-binding protein. Table V lists the existing NFAT members. For simplicity, in this chapter, I would be referring the NFAT members as NFATc1 to NFATc5. All five NFAT proteins have a common Rel-homology domain and recognize similar DNA binding sites in the regulatory regions of numerous genes (Rao, Luo, and Hogan 1997a). Interestingly, the NFAT family and Rel/NFκB family are evolutionary related despite sharing a marginal ~17% sequence identity, however, their structural similarity is remarkable. It should be noted that the molecular weight of NFAT proteins ranges from 120-140kDa.

Table IX : List of NFAT family members. From (Hogan et al. 2003)

<table>
<thead>
<tr>
<th>Members</th>
<th>Other names</th>
<th>Regulation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFAT1</td>
<td>NFATc2; NFATp</td>
<td>Ca(^{2+})/calcineurin</td>
<td>(McCaffrey, Goldfeld, and Rao 1994)</td>
</tr>
<tr>
<td>NFAT2</td>
<td>NFATc1; NFATc</td>
<td>Ca(^{2+})/calcineurin</td>
<td>(Northrop et al. 1994)</td>
</tr>
<tr>
<td>NFAT3</td>
<td>NFATc4</td>
<td>Ca(^{2+})/calcineurin</td>
<td>(Hoey et al. 1995)</td>
</tr>
<tr>
<td>NFAT4</td>
<td>NFATc3; NFATx</td>
<td>Ca(^{2+})/calcineurin</td>
<td>(Hoey et al. 1995; E. S. Masuda et al. 1995)</td>
</tr>
<tr>
<td>NFAT5</td>
<td>TonEBP</td>
<td>Osmotic stress</td>
<td>(Miyakawa et al. 1999; López-Rodríguez et al. 1999; Jauliac et al. 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Integrin (α6β4) activation</td>
<td></td>
</tr>
</tbody>
</table>

**Discovery and tissue distribution of NFAT family**

NFAT was identified as a rapidly inducible nuclear factor binding to the distal antigen receptor response element, ARRE-2, of the human IL-2 promoter in T cells (Shaw et al. 1988). Calcium mobilization and the activation of calcineurin results in the appearance of NFAT in the nucleus. It should be noted that the molecular weight of NFAT proteins ranges from 120-140kDa. The members of the NFAT family differed in the way they were isolated. For instance, the cDNA clones encoding NFATc3 and NFATc4, were isolated from Jurkat T cell and human peripheral blood lymphocyte (PBL) cDNA libraries via cross-hybridization to an NFATc2 probe. Interestingly, cDNA clones encoding NFATc3 gene were also isolated from murine thymus cDNA libraries (Rao, Luo, and Hogan 1997a).

The expression of NFAT genes can be found in almost all tissues, however, there is a variation in the expression patterns among different NFAT family members. Except NFATC4, all of the NFAT genes have a strong expression in the immune system, in the thymus, spleen, and peripheral blood lymphocytes as well as lower expression levels in other tissues (Figure 40).
Figure 40: Tissue distribution of different NFAT genes (NFATC1, -C2, -C3, and -C4) which was generated using a public database known as the human protein atlas. It shows tissue distribution of different NFAT genes based on the RNA-seq tissue data generated by the Genotype-Tissue Expression (GTex) project (another public database). From (Uhlén et al. 2015)

4.2 Physiological roles of NFATs

NFAT genes (termed NFATc genes), which are found only in the genomes of the vertebrates, are thought to be arisen following the recombination of an ancient precursor with a Rel domain about 500 million years ago. It has been proposed that Ca$^{2+}$ signals were redirected to a new transcriptional program via this recombination and provided part of the groundwork for vertebrate morphogenesis and organogenesis. In the following paragraphs, I would be briefly describing the roles of NFATs in different organs (Hai Wu et al. 2007).
Immune system

NFAT signaling is implicated in different phases of development and functions of the immune system. For instance, NFATc1, NFATc2, and NFATc3 control the expression of a variety of cell membrane and secreted proteins, thereby making them crucial for T cell activation by antigen. Further, NFAT also participates in T cell differentiation by controlling transcription factors such as T-bet, a master regulator of Th1 (T-helper 1) cell development (Peng, Szabo, and Glimcher 2002). Interestingly, NFATc4 is not expressed in T cells and the role of NFATc5 in T cell functions has not yet been underlined (I. A. Graef, Chen, and Crabtree 2001).

NFATs are essential for the expression of cytokine genes. Interestingly, it was shown that transcription factor Foxp3 partners with NFAT and suppresses their transcriptional activity. This suppression promotes generation of T regulatory cells and inhibition of cytokine production and T cell effector function. Therefore, this implicates NFAT signaling in the function of suppressor cells (Bettelli, Dastrange, and Oukka 2005; Y. Wu et al. 2006). Additionally, NFAT signaling is required in early development for the actions of the pre-TCR (T cell receptor) whereas in the late stages of development, it is required for the positive selection of a repertoire of T cells which are able to respond to antigens presented by the MHC molecule. Finally, calcineurin and NFATc1 are crucial for immunogenic responses and the formation of B1 cells in B cells (Berland and Wortis 2003; Winslow, Gallo, et al. 2006). Table X summarizes the phenotypes in the immune system observed in NFAT-deficient mice.

Table X Phenotype in the immune system in NFAT-deficient mice. TH-2: Thelper-2 cells; IL-4: Interleukin-4; TCR: T-cell receptor. From (Macian 2005)

<table>
<thead>
<tr>
<th>NFAT protein</th>
<th>Phenotype in the immune system</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFATc1</td>
<td>Reduced proliferative responses by T cells; Improved TH2-cell responses and IL-4 production</td>
<td>(Ranger, Hodge, et al. 1998; Peng et al. 2001)</td>
</tr>
<tr>
<td>NFATc2</td>
<td>Moderately enhanced B- and T-cell responses; prolonged IL-4 expression; delayed thymic involution</td>
<td>(Yoshida et al. 1998; Schuh et al. 1998; Kiani et al. 2001)</td>
</tr>
<tr>
<td>NFATc1 and NFATc2</td>
<td>Grossly impaired T-cell effector functions in fetal liver chimeras with profound defects in cytokine production; B-cell hyperactivity</td>
<td>(Ranger, Oukka, et al. 1998)</td>
</tr>
<tr>
<td>NFATc3</td>
<td>Impaired development of CD4 and CD8 single-positive cells with increased apoptosis of double-positive thymocytes</td>
<td>(Oukka et al. 1998)</td>
</tr>
<tr>
<td>NFATc2 and NFATc3</td>
<td>TCR hyper-activity with profound lymphoproliferative disorder; notable increase in TH-2 cell responses.</td>
<td>(Starr, Jameson, and Hogquist 2003; Rengarajan, Tang, and Glimcher 2002)</td>
</tr>
</tbody>
</table>
**Nervous system**

NFAT family plays a critical role in normal and pathological nervous system. During the development of mammalian nervous system, NFAT signaling was reported to be a major player in corticogenesis, synaptogenesis and neuritogenesis.

Corticogenesis is the process in which the cerebral cortex of the brain is formed during the development of the nervous system. In corticogenesis, the gene transcription is regulated by store-operated Ca²⁺ entry (SOCE) activation which mediates the neural progenitor cell proliferation via calcineurin/NFAT signaling. Interestingly, NFATc3 plays a major role in this process and is the predominant NFAT gene in neural progenitor cell cultures. It is also a potent inducer of neural progenitor cell differentiation into neurons and astrocytes (Somasundaram et al. 2014; Serrano-Pérez et al. 2015). Furthermore, in the developing brain of rats and traumatic brain injury models, differential expressions of NFATc3 and NFATc4 were reported (Ulrich et al. 2012). These observations suggested that in the damaged and growing nervous tissue, different NFATs are employed at the same time and that characterizing the effects of such changes in NFAT might provide new therapeutic targets for neurodevelopmental disorders (Kipanyula, Kimaro, and Etet 2016).

Synaptogenesis is the formation of synapses between neurons in the nervous system. Synaptogenesis requires the complex interactions between the activities of the receptors of two neurotransmitters: the inhibitory gamma-aminobutyric acid (GABA) and the excitatory N-methyl-D-aspartate receptor (NMDA) activities. Importantly, this interaction, through the calcineurin-dependent transcription of brain-derived neurotrophic factor (BDNF), plays an important role in the induction of immediate early genes which are necessary for effective changes in synaptic plasticity and long-term memory formation (Fukuchi et al. 2014; Tsuda 2015; Fukuchi et al. 2015; Niwa et al. 2012).

**Heart, skeletal muscle, and bones**

NFAT family members have been found to function in both endocardium and the myocardium in the morphogenesis of heart valves. NFAT signaling is essential for myocardial growth and responses to hypertrophic stimuli through which it contributes in the development of the myocardium, an ancient muscle type (Molkentin et al. 1998). NFAT activity has been known to play critical roles in the embryonic development and for adult adaptation to cardiac stress (Crabtree and Olson 2002).

In vertebrates, skeletal muscle contains myofibers with specific contractile, metabolic and structural properties that includes slow and fast fibers essential for complex contractile responses (Parsons et al. 2004). NFAT signaling is involved in maintaining the balance in the proportion of slow relative to fast myofibers within skeletal muscle. Moreover, as muscles from vertebrates are bulkier than those of invertebrates, these bigger myofibers fuse with other myocytes and thus increase their size. It has been observed that NFATc2 controls this myoblast fusion with multinucleated myotubes and facilitates the regulation of skeletal muscle growth (Horsley et al. 2003). Therefore, altogether, the ability of vertebrate muscles to grow at a faster rate and beyond a certain physical size, is enabled by NFAT signaling (Hai Wu et al. 2007).
NFAT proteins control the formation of bones by functioning in both osteoblasts and osteoclasts. NFATc1 functions downstream of a vertebrate-specific receptor, receptor activator of nuclear factor κB ligand (RANKL) in the osteoclasts of vertebrates. Indeed, NFATc1 is used by RANKL to specify the fate of osteoclast and osteoblasts are absent in NFATc1 mutant mice (F. Ikeda et al. 2004). Further, NFATc1 plays a critical role in the proliferation of osteoblasts. It also facilitates the regulation of factors that are crucial to the recruitment of osteoclast precursors to bone (Koga et al. 2005; Winslow, Pan, et al. 2006). Therefore, NFAT signaling participates in the development of this system by coordinating the activities of two major cell types of bones.

4.3 NFAT and disease

For more than 25 years, NFAT functions have been targeted to develop drugs such as CsA and FK506 for controlling T cell immunity in autoimmune diseases. Interestingly, in addition to autoimmune diseases, research from various groups have highlighted the importance of NFAT family members in diseases such as cancer, neurodegenerative diseases, and cardiovascular diseases. In this section, I would describe briefly how NFATs contribute to the pathogenesis.

Autoimmune Diseases

The deregulation of Ca^{2+}/NFAT signaling in autoreactive T cells has been involved in many autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjogren’s syndrome (SS), and psoriasis (J.-U. Lee, Kim, and Choi 2018; Park et al. 2020). RA is a chronic systemic inflammatory disease. Evidence suggest that synovium has presence of autoreactive T cells in RA patients (Firestein 2003) and the synovium has high NFAT expression (K. Masuda et al. 2002). Rise in intracellular Ca^{2+} levels lead to an increase in the calcineurin activity in synoviocytes of RA patients. This activates the NFAT signaling pathway, which in turn, induce the expression of pro-inflammatory cytokines such as IL-6 and matrix metalloproteinases (MMPs). Therefore, NFAT signaling plays an important role by promoting the production of these cytokines and MMPs in various types of synovial inflammatory cells of RA patients (Park et al. 2020).

Involved in multiple organs, SLE leads to tissue damage to any part of the body with diverse clinical symptoms. The major immunologic perturbance in SLE is autoantibody production such as anti-SSA, anti-SSSB, anti-double-strand DNA, anti-Sm, and anti-ribonucleoprotein. This results from a generalized immune cell dysfunction that promotes B cell hyperactivity. In SLE patients, B cell stimulation showed an increase in intracellular Ca^{2+} response compared to healthy controls (Liössis et al. 1996). In animal models, high Ca^{2+} responses in B cells were in line with developed autoimmune symptoms that resembled SLE (Hibbs et al. 1995). Studies in SLE patients suggest that the Ca^{2+}/calcineurin/NFAT axis is abnormally activated in the T and B cells which breaks B cell tolerance and induces T cell differentiation towards pathogenic Th17 subtype (Crispin et al. 2008). Therefore, this pathway can be a therapeutic target in SLE patients (Park et al. 2020).
Cancer

The dysregulation of the NFAT signaling pathway has been found to be associated with several cancers such as breast cancer (Yoeli-Lerner et al. 2005; 2009), pancreatic cancer (Baumgart et al. 2012), Burkitt’s lymphoma (Marafioti et al. 2005), and aggressive T cell lymphoma (Fu et al. 2006). NFATs regulate cellular functions such as cancer cell proliferation, apoptosis suppression, induction of invasion and migration, and drug resistance through calcineurin-dependent and -independent pathways (Qin et al. 2018).

Table XI displays a summary of regulation of NFAT in cancer. For example, with an overall 5-year survival rate of <11%, malignant melanoma (MM) is the most notorious form of skin cancer which is in need of new therapeutic strategies (Dhomen and Marais 2007). Almost 50-70% of all melanomas contain mutations in BRAF gene (most commonly a BRAFV600E) which is the driving factor in melanomagenesis in mice (Davies et al. 2002; Dhomen and Marais 2007). Flockhart et al., demonstrated that human metastatic melanoma cell lines have a high expression of NFATs (NFATc1 and NFATc3) and they are activated by oncogenic BRAFV600E via the canonical MEK/ERK signaling. Furthermore, using CaN inhibitors and silencing NFATc1 and NFATc3, it was observed that NFAT was required for COX-2 promoter activation and protein induction in metastatic melanoma cells. Therefore, as COX-2 stimulates cell proliferation, and tumor metastasis (Buchholz and Ellenrieder 2007), the NFAT/COX-2 signaling could be targeted for therapy in melanoma (Flockhart et al. 2009).

Table XI : Summary of regulation of NFAT members in cancer. From (Pan, Xiong, and Chen 2013)

<table>
<thead>
<tr>
<th>NFAT member</th>
<th>Cell/Tissue type</th>
<th>Tumor/Phenotype</th>
<th>Mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFATc1</td>
<td>3T3-L1 fibroblasts</td>
<td>Transformed phenotype</td>
<td>Stimulation of c-Myc expression and JAK-Stat pathway activation</td>
<td>(Neal and Clipstone 2003; Lagunas and Clipstone 2009)</td>
</tr>
<tr>
<td>NFATc2</td>
<td>Breast carcinoma cell line</td>
<td>Phenotype of enhanced invasive migration</td>
<td>Activation of JNK and p38MAPK via GPC6 and Wnt5A signaling</td>
<td>(Foldynová-Trantírková et al. 2010)</td>
</tr>
<tr>
<td>NFATc1 and NFATc3</td>
<td>A375, CHL-1, and WM266-4</td>
<td>Human melanoma cell lines</td>
<td>Upregulation of NFATc1 and -c3 by the oncogenic BRAF mutation through MEK/ERK signaling</td>
<td>(Flockhart et al. 2009)</td>
</tr>
<tr>
<td>NFATc4</td>
<td>C141 epidermal cells</td>
<td>Transformed phenotype</td>
<td>Enhanced expression of Cox-2</td>
<td>(Y. Yan et al. 2006)</td>
</tr>
</tbody>
</table>

Neurodegenerative diseases

Parkinson’s disease (PD) has been described in the previous chapter on TRIM proteins. So far, the molecular mechanisms and the intracellular signaling pathways by which α-synuclein contributes to neuronal death in PD remains poorly understood. RNA sequencing analysis have shown that
NFATc3 is the predominant NFAT member expressed in mouse SN DA neurons (substantia nigra dopaminergic) isolated by laser capture microdissection and in whole brain (J. Luo et al. 2014). Two independent studies have implicated NFATc3 in α-synuclein-induced degeneration of midbrain DA neurons (J. Luo et al. 2014; Caraveo et al. 2014). They suggest that the cytosolic calcium levels that drives a calcium/calcineurin/NFATc3 cascade is enhanced by α-synuclein and results in the activation of a neurotoxic program. Consequently, inhibition of calcineurin has been shown to block the nuclear translocation of NFATc3 and to improve the survival of primary DA neurons from transgenic mice expressing a pathogenic form of α-synuclein (J. Luo et al. 2014). Therefore, a direct targeting of NFATc3 might represent a more specific and promising therapeutic strategy.

NFAT signaling has also been implicated in Alzheimer’s disease (AD-mentioned in the previous chapter). Abdul et al., 2009 suggested that in neurodegenerative diseases, the alterations in NFAT signaling are selective and such selective alterations might play a key role in Aβ-induced neurodegeneration. Amyloid beta (Aβ) peptides are defined as the main component of the amyloid plaques found in the brains of AD patients and are crucially implicated in AD. An increase in calcineurin A activity and nuclear translocation of NFATc2 and NFATc4 in human hippocampus with increased dementia severity was observed. NFATc2 was found to be more active in AD patients with mild cognitive impairment whereas AD patients with severe dementia had a high expression of NFATc4. Interestingly, the same study found that changes in calcineurin/NFATc3 were directly correlated to soluble Aβ levels in postmortem hippocampus (Abdul et al. 2009).

Altogether, NFAT signaling is implicated in various pathologies and therefore, it is important to understand their regulation which might facilitate the direct targeting of either NFAT members or their regulators for therapy with minimal side effects.

Figure 41: Primary structure of NFAT composed of different domains such as N- and C-terminus transactivation domains (TAD-N and C terminus), NFAT homology region (NHR), and DNA binding domain (DBD). The NHR contains two calcineurin-binding sites: Serine Rich Region (SRR1 and SRR2) and Serine Proline region (SP1, SP2, and SP3) which docks 14 phosphoserine residues. It also contains nuclear localization signal (NLS) and nuclear export signal (NES). Another NLS can also be found in the DBD. The TAD-C region of NFATc1 and NFATc2 has two sumoylation sites. Different transcription factors and proteins cooperates with NFAT in specific domains in NFAT and they are listed above each NFAT domain. From (Mognol et al. 2016a)
4.4 Structure and functional domains of NFATs

The conserved regions of calcium-regulated NFAT proteins consist of two tandem domains: (1) a regulatory domain namely NFAT-homology region (NHR); and (2) the DNA binding region (also known as Rel-homology region, RHR). Figure 41 gives a schematic representation of the NFAT primary structure. Different NFAT members share a moderate 22-36% sequence identity in the NHR region. The DBD is ~270 amino acids long region and shares 64-72% sequence identity among the different NFAT members. The NHR and DBD domains are flanked by two highly variable N and C-terminal transactivation domains (TAD).

Transactivation Domains (TAD)

NFAT do not act as transcription factors alone but mostly co-operate with other transcription factors such as activator protein1 (AP-1), GATA3, Foxp3, etc. TADs are the putative sites for the interaction of distinct NFAT members with specific partners (Figure 41) and thereby control key biological functions such as proliferation and cell death. NFAT is composed of two TAD domains at the N-terminus and C-terminus, respectively. Furthermore, K residues in the TADs of NFAT are modified by PTMs particularly SUMOylation. For example, the well conserved Lys (K) residue in the TAD of NFATc1 and NFATc2 is SUMOylated which contributes to their nuclear localization and transcriptional function (Terui et al. 2004; Nayak et al. 2009). Therefore, this region might be important for the function mediated by different NFATs (Mognol et al. 2016b).

NFAT Homology Region (NHR)

The NFATc1-NFATc4 family members share a N-terminal regulatory domain also referred to as NFAT homology region (NHR). This moderately conserved region is ~300 amino acid long and is located just N-terminal to the DNA Binding Domain (DBD). In NFATc2, mass spectrometric analysis revealed conserved sequence motifs namely SRR1, SP2, SP3, and SRR2 which are phosphorylated at 14 phosphoserine residues in resting cells. These motifs are dephosphorylated by calcineurin (CaN), a calcium/calmodulin-dependent serine/threonine phosphatase, leading to the nuclear accumulation of NFAT (Hogan et al. 2003). Interestingly, mutations of serine-to-alanine in these motifs of NFATc2 suggested that the N-terminal SRR1 motif (Fig. 28) is a critical region whose dephosphorylation controls the exposure of a nuclear localization signal (NLS) in the regulatory domain (Kipanyula, Kimaro, and Etet 2016).

DNA Binding Domain

The DNA-binding domains (DBD) also known as Rel-Homology region (RHR) of NFAT, in the three-dimensional structure, are similar to the DNA-binding domains of Rel-family proteins (Jain et al. 1995; Nolan 1994; Chytíl and Verdine 1996). DBDs are ~300 amino acid long and are highly conserved within each family with 70% pairwise identity for NFAT proteins and 41-61% identity for mammalian Rel proteins (Hoey et al. 1995). Indeed, majority of the base-specific contacts with DNA
is made by this region. This domain contains highly conserved RAHYETEG sequence in which the underlined residues are likely to contact DNA (Jain et al. 1995; Chytíl and Verdine 1996). NFAT and Rel proteins differ in their structures in solution. NFAT proteins are monomeric in solution as well as when bound to DNA whereas Rel proteins are dimeric in solution and bind DNA as dimers.

**C-terminal domain**

As a result of splicing events, multiple isoforms of NFAT proteins have a variable C-terminal domain that could influence their protein-protein interactions, and the post-translational modifications that regulate their stability and localization. For example, in a previous study we have shown that NFATc3 could be SUMOylated on three different sites (K435, K704, and K1013) (Figure 42). Importantly, Trim17 interacted with NFATc3 in a SUMO-dependent manner whereas its interaction with NFATc4 was SUMO-independent (Mojsa et al. 2015a). This difference in SUMO-dependent/independent interaction of Trim17 with NFATc3 and NFATc4 might be due to the lack of the third SUMO consensus site, K1013, in NFATc4.

![Table: SUMOylation consensus motifs of murine NFATc3 and corresponding sequences in murine NFATc1, NFATc2, and NFATc4. Bold amino acids represent SUMOylation motifs while the sequences identical to NFATc3 are underlined (Mojsa et al. 2015a).](image)

**4.5 NFAT and transcription**

NFAT are known to cooperate with other transcription factors in the nucleus to activate their downstream target genes. These transcription factors include activator protein-1 (AP-1: Fos/Jun), GATA-2/3/4, Foxp3, MEF2, CBP/p300 etc. (Macián, López-Rodríguez, and Rao 2001; Mognol et al. 2016a). I would be briefly describing above mentioned partners of NFAT and their target genes in this section.

**NFAT and Partners**

NFAT:AP-1 : AP-1 transcription factors that bind as homo- or heterodimers to the AP-1 regulatory elements present in many genes are defined as the basic region-leucine zipper (bZIP) proteins of the Jun (cJun, JunB and JunD) and Fos (cFos, FosB,Fra1 and Fra2) families (Glover and Harrison 1995).
In immune cells, the cooperation between NFAT and AP-1 transcription factors has been shown in the promoter/enhancer region of several genes such as *IL-2*, *IL-4*, *IL-5*, *IFN-γ*, *FasL*, etc. (Rao, Luo, and Hogan 1997b). Interestingly, a wide range of composite binding sites have been observed (Figure 43), and *in vivo*, a comparable degree of cooperativity appears to be achieved by pairing weak NFAT sites with moderately strong AP-1 sites (Rao, Luo, and Hogan 1997b).

![Composite NFAT/AP-1 sites](image)

Figure 43: Representation of NFAT/AP-1 binding site. The nucleotides in the core of the site are represented in bold. From (Macián, López-Rodríguez, and Rao 2001)

The contacts for this complex involve the amino-terminal domain of the NFAT homology region, however, the carboxy-terminal domain of this region is also involved in some contacts with Fos too (L. Chen et al. 1998). Interestingly, in the previous study, we had identified a region around the transcription start site of *Trim17* promoter (a target gene and regulator of NFATc3) which contained two conserved AP-1 binding sites and one conserved composite NFAT:AP-1 element. Indeed, it was demonstrated that NFATc3 induced the transcription of *Trim17* in association with c-Jun in early apoptotic neurons (Figure 44) (Mojsa et al. 2015a).

![Alignment of the mouse and human genomic sequences around the transcription start site of mouse Trim17.](image)

Figure 44: Alignment of the mouse and human genomic sequences around the transcription start site of mouse Trim17. The region shown is included entirely in exon1 of human TRIM17. * indicate identical nucleotides. The boxed region indicates the two potential AP-1 binding sites and one potential composite NFAT-AP-1 site that are conserved between mouse and human (Mojsa et al. 2015a).
NFAT:GATA: NFAT family has also been shown to partner with the GATA transcription factor family composed of four members (GATA-1,-2,-3,-4). It is, however, not clear whether this is a result of a physical mechanism of cooperative stabilization of NFAT-GATA complexes on DNA. A Y2H assay has revealed that NFATc4 and GATA4 indeed interact with each other, and this interaction might be important for cardiomyocyte growth and cardiac hypertrophy. Further, NFATc1 and GATA2 are co-expressed in nuclei of developing myoblasts differentiating in response to IGF-1, and could be co-immunoprecipitated from skeletal muscle extracts (Macián, López-Rodríguez, and Rao 2001). This partnership is observed in a large number of different cell types (Macián, López-Rodríguez, and Rao 2001).

Table XII Partners of NFAT in transcription. Abbreviations included are ND: non determined; IL: interleukins; TNF-α: Tumor necrosis factor- α; BNP: brain natriuretic peptide; Nur77: nerve growth factor IB; BDNF: brain derived nerve growth factor. From (Mognol et al. 2016)

<table>
<thead>
<tr>
<th>Partner</th>
<th>NFAT member</th>
<th>Region of interaction on NFAT</th>
<th>Effect on transcription</th>
<th>Gene regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1 (c-Fos/c-Jun)</td>
<td>NFATc1;c2;c3;c4</td>
<td>DBD; TAD-C</td>
<td>Activation</td>
<td>Several cytokines IL-2</td>
</tr>
<tr>
<td>CBP/p300</td>
<td>NFATc1-β; NFATc2-C; NFATc4-A</td>
<td>TAD-N; NHR</td>
<td>Activation</td>
<td>IL-2; TNF-α</td>
</tr>
<tr>
<td>ERα/ERβ</td>
<td>NFATc4-A</td>
<td>TAD-N;NHR</td>
<td>Inhibition</td>
<td>IL-2</td>
</tr>
<tr>
<td>GATA-2</td>
<td>NFATc1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GATA-3</td>
<td>NFATc1-α; NFATc2-C</td>
<td>ND</td>
<td>Activation</td>
<td>BNP</td>
</tr>
<tr>
<td>GATA-4</td>
<td>NFATc4</td>
<td>NBD</td>
<td>ND</td>
<td>IL-5</td>
</tr>
<tr>
<td>MEF2D</td>
<td>NFATc2-C</td>
<td>TAD-C</td>
<td>Activation</td>
<td>Nur77</td>
</tr>
</tbody>
</table>

NFAT:CBP/p300: The CBP-p300 coactivator family is composed of two closely related transcriptional co-activating proteins (or co-activators): CREB-binding protein (CBP or CREBBP) and p300 (also referred to as EP300 or E1A binding protein p300). It was shown that CBP and p300 could directly interact with NFATc1 and NFATc2 (C. García-Rodríguez and Rao 1998; Avots et al. 1999).

NFAT:MEF2: The transcription factors MEF2 have diverse functions in a wide range of tissues such as muscle, cardiac, skeletal, vascular, etc. (Pon and Marra 2015). It interacts with C-terminal of TAD of NFATc2 isoform C (H.-D. Youn, Chatila, and Liu 2000), however, it does not interact with NFATc1-α nor NFATc4-A (Fig. 30) (Blaeser et al. 2000). Further, the interaction between NFATc2 and MEF2 led to a synergistic activation of pathways involved in cell death, muscle development (Blaeser et al. 2000; H. Wu et al. 2000), thymocyte-negative selection, and apoptosis (H.-D. Youn, Chatila, and Liu 2000; Blaeser et al. 2000).
Target genes of NFAT

NFAT have different physiological roles. Together with their transcriptional partners described above they regulate different cellular functions that include apoptosis, proliferation, transcription, etc. by controlling the expression of their target genes. Depending on the cellular context, NFAT can either act as activators or repressors of transcription. In the immune system, NFAT act as transcriptional activators by coordinating the expression of several cytokine genes such as TNFα, IL-2 (T. N. Nguyen et al. 2010; C. García-Rodríguez and Rao 1998; Avots et al. 1999; T. Yang, Davis, and Chow 2001), and IL-4 (I.-C. Ho et al. 1996). Furthermore, NFAT control the expression of cell cycle genes such as cyclins A2, B1, E and F (Caetano et al. 2002). Interestingly, NFATc1 upregulates cyclin A2 expression (Karpurapu et al. 2008) whereas NFATc2 downregulates the expression of cyclin A2 (Carvalho et al. 2007). Additionally, NFAT dependent transcription is crucial for the expression of several pro-apoptotic genes such as FasL (Latinis et al. 1997; Holtz-Heppelmann et al. 1998), Trim17 (Irina Lassot et al. 2010a), Nur77 (H.-D. Youn, Chatila, and Liu 2000; H. D. Youn and Liu 2000), TRAIL (Q. Wang et al. 2011), etc. and anti-apoptotic genes such as Bcl2 (Kawamura et al. 2004; Patra et al. 2013), BDNF (Quadrato et al. 2012; Mojsa et al. 2015a), etc. Interestingly, NFATc4 acts as a repressor for growth associated protein gene (GAP-43) during neuronal maturation (T. Nguyen et al. 2009). Table XII and Table XIII summarizes the partners of NFAT family, region of interaction, the effect on transcription as well as the genes regulated by NFAT with respect to different cellular functions, respectively (Mognol et al. 2016b).

4.6 Regulation of NFAT

Calcium signaling affects every cell at some point in their growth, differentiation or function. Many external signals cause an increase in cytoplasmic Ca\(^{2+}\) levels. Upon this increase, Ca\(^{2+}\) binds to its
most common cognate receptor calmodulin (a small calcium-binding protein that interacts and regulates the activity of numerous proteins), and activates one of the three major pathways. Two of these pathways result in the activation of the transcriptional regulator, CREB while in the third pathway, Ca$^{2+}$-calmodulin activates calcineurin that, among other targets, dephosphorylates NFAT and allows their translocation to the nucleus (Putney 2012). The calcineurin/NFAT signaling pathway is involved in numerous cellular processes and its dysregulation results in various pathological diseases such as neurodegenerative diseases, autoimmune diseases, and cancer. Therefore, understanding the regulation of NFAT is of utmost importance.

**The calcineurin/NFAT signaling pathway**

The calcineurin/NFAT signaling pathway is unique to vertebrates and it is known to orchestrate critical cellular interactions that characterize vertebrate development and morphogenesis (Klee, Ren, and Wang 1998; Hogan et al. 2003; Hai Wu et al. 2007). Calcineurin (PP2B) is defined as a calcium/calmodulin-dependent serine/threonine phosphatase and it is implicated in numerous biological processes. It is a heterodimer composed of a 59-62kDa catalytic subunit A (CnA) and a 19kDa regulatory subunit B (CnB) (Medyouf and Ghysdael 2008).

In resting cells, NFAT proteins reside in the cytoplasm in a hyperphosphorylated, inactive form (C. Luo et al. 1996; Beals, Clipstone, et al. 1997; H. Okamura et al. 2000). Their activation is induced after the engagement of cell surface receptors such as T-cell receptors (TCR), receptor tyrosine kinases (RTKs), and G-protein coupled receptors (GPCRs) (Serafini et al. 1995; Crabtree and Olson 2002). Once engaged or ligated, these receptors activate phospholipase C$_\gamma$ (PLC$_\gamma$). This results in
the cleavage of membrane bound phosphatidylinositol 4,5-bisphosphate (PIP2) and the release of diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) (Gwack et al. 2007). In the endoplasmic reticulum (ER), IP3 binds to the IP3 receptors resulting in the release of Ca\textsuperscript{2+} from intracellular storage sites that triggers the opening of specialized store-operated calcium channels (SOC) (Gwack et al. 2007; Kar, Nelson, and Parekh 2011). Now, the free intracellular calcium binds to calmodulin which further binds to calcineurin. Once calcineurin is activated, it dephosphorylates NFAT and results in its nuclear translocation. Once in the nucleus, NFAT binds to the target gene promoter through homo- or heterodimerization, or co-operation with other partners such as transcription factors AP-1, GATA4, MEF2, etc. (described in the next section). Depending on the partner, it either induces or represses the transcription of its downstream target genes (Figure 45) (Hogan et al. 2003; Gwack et al. 2007; Medyouf and Ghysdael 2008; Kar, Nelson, and Parekh 2011; Choo, Yeo, and Zayzafoon 2009; Qin et al. 2014).

**Regulation of NFAT by kinases**

The nuclear/cytoplasmic shuttling of NFAT is an important mechanism for the regulation of transcriptional activity of NFAT. Indeed, several priming, export, and maintenance NFAT kinases such as protein kinase A (PKA) (Gwack et al. 2006), dual-specificity tyrosine-phosphorylation regulated kinase 1a (DYRK1a) (Gwack et al. 2006), glycogen-synthase kinase 3\textbeta\ (GSK3\textbeta\) (Beals, Sheridan, et al. 1997), and casein kinase 1 (CK1) (Heidi Okamura et al. 2004), tightly control the balance between nuclear import/export of NFAT proteins. The nuclear translocation of NFAT proteins is facilitated by the export kinases whereas the maintenance kinases retain the hyperphosphorylated form of NFAT proteins in the cytoplasm. Table XIV enlists the NFAT kinases involved in the signaling (M. R. Müller and Rao 2010).

Indeed, when the intracellular calcium levels are low, a highly effective nuclear export mechanism composed of nuclear priming kinases such as DYRK1a and PKA abolish the import of nuclear NFAT proteins (Gwack et al. 2006). Interestingly, DYRK1a phosphorylates nuclear NFAT which creates substrate sites (i.e. priming) for the subsequent rephosphorylation by GSK3\textbeta\ and nuclear export (Gwack et al. 2006). Furthermore, several maintenance kinases such as CK1, mitogen activated protein kinases (MAPKs), c-JUN kinase (JNK), and extracellular signal related kinase (ERK) control the retention of NFAT in the cytosol (Porter, Havens, and Clipstone 2000; Zhu et al. 1998; T. T. C. Yang et al. 2002; M. R. Müller et al. 2009; C. W. Chow et al. 1997; Ortega-Pérez et al. 2005; Y.-C. Lee et al. 2012). For example, in NFATc4, the Ser168 and Ser170 are phosphorylated by P38 MAPKs whereas the CaN targeting domain in NFATc1 and NFATc3 is phosphorylated by JNK kinases (Yoeli-Lerner et al. 2005; C.-W. Chow et al. 2000).

**Regulation by Ubiquitination**

In contrast to the regulation of NFAT by kinases which has been the subject of interest of many laboratories, regulation of NFAT by the UPS has been poorly studied. Only few studies have addressed this point and even fewer studies have identified the plausible E3 ubiquitin ligases of NFAT. Table XI summarizes the studies done on understanding NFAT regulation by the UPS.
Table XIV: List of NFAT kinases involved in the signaling. CK1: Casein Kinase1; DYRK: dual specificity tyrosine-phosphorylation regulated kinase; GSK3β: glycogen synthase kinase 3β; SP: Ser-Pro-X-X repeat motif; SRR: serine-rich region. From (M. R. Müller and Rao 2010)

<table>
<thead>
<tr>
<th>NFAT kinase</th>
<th>Type of kinase</th>
<th>Substrate</th>
<th>Site of phosphorylation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK3β</td>
<td>Export</td>
<td>NFATc1</td>
<td>SP2 and SP3</td>
<td>(Beals, Sheridan, et al. 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NFATc2</td>
<td>SP2</td>
<td></td>
</tr>
<tr>
<td>CK1</td>
<td>Export and maintenance</td>
<td>NFATc2</td>
<td>SRR1</td>
<td>(Heidi Okamura et al. 2004)</td>
</tr>
<tr>
<td>DYRK1</td>
<td>Export</td>
<td>NFATc1 and NFATc2</td>
<td>SP3</td>
<td>(Arron et al. 2006; Gwack et al. 2006)</td>
</tr>
<tr>
<td>DYRK2</td>
<td>Maintenance</td>
<td>NFATc1 and NFATc2</td>
<td>SP3</td>
<td>(Arron et al. 2006; Gwack et al. 2006)</td>
</tr>
</tbody>
</table>

For instance, several studies have investigated the ubiquitination/degradation of NFATc1, which plays an important role in osteoclastogenesis. Kim et al., 2010 showed that the E3 ubiquitin-ligases Cbl-b and c-Cbl increase the ubiquitination and induce the degradation of NFATc1 in a Src kinase-dependent manner during late stage osteoclastogenesis. Overexpression of c-Src induced the down-regulation of NFATc1 while the degradation of NFATc1 was blocked by the depletion of Cbl proteins (J. H. Kim et al. 2010). Importantly, the expression of Nuclear receptor77 (Nur77), which is transcriptionally induced by NFATc1 at the late stage of osteoclast differentiation, in turn, transcriptionally up-regulates Cbl-b which further induces NFATc1 degradation, thereby creating a negative feedback loop (Li et al. 2015). Youn et al., 2012 demonstrated that the protein stability of NFATc1 was negatively regulated by JMJD5 (a Jumonji C {Jmjc} domain-containing protein). JMJD5 was found to hydroxylase NFATc1 protein through its enzymatic activity and it induced the association of hydroxylated NFATc1 with the E3 ubiquitin ligase Von Hippel-Lindau tumor suppressor (VHL). This might facilitate the proteasomal degradation of NFATc1 via ubiquitination (M.-Y. Youn et al. 2012). Additionally, Narahara et al., 2019 showed that the E3 ubiquitin ligase, Cullin3 together with kelch repeat and BTB domain-containing protein 11 (KBTBD11- a member of the KBTBD subfamily of proteins) promoted the ubiquitination and degradation of NFATc1 by the proteasome. Interestingly, silencing of KBTBD11 increased the expression of NFATc1 whereas overexpression of KBTBD11 had an opposite effect. Moreover, it was shown that degradation induced by Cullin3 decreased the differentiation of osteoclasts. Therefore, some of these studies demonstrated that the regulation of NFATc1 by the UPS can have a direct impact on the physiological function (Narahara et al. 2019).

As phosphorylation of NFAT is an integral part of NFAT regulation, there have been studies which have observed a cross-talk between phosphorylation and ubiquitination pertaining to regulation of NFAT. Yoeli-Lerner et al. 2005, had observed that activation of Akt/PKB (protein kinase B) in breast cancer cells inhibited carcinoma migration and invasion by inhibiting the transcriptional activity of NFAT. Interestingly, they found that signaling via Akt reduced NFAT expression levels due to ubiquitination and proteasomal degradation mediated by the E3 ubiquitin ligase HDM2 (the human
homolog of murine double minute 2). Therefore, they concluded that Akt can block the cell motility and invasion in breast cancer cells by a mechanism that depends in part on NFATc2 (Yoeli-Lerner et al. 2005). Yoeli-Lerner et al. 2009 further demonstrated that activation of Akt results in the inactivation of GSK-3β (a major effector of Akt) which ultimately leads to the degradation of NFAT by the proteasome, and consequently cell migration inhibition (Yoeli-Lerner et al. 2009).

Additionally, Singh et al. 2011 further studied the regulation of NFATc2 by HDM2. Interestingly, they found that zoledronic acid induces HDM2 in cancer cells. The overexpression of HDM2 led to a dose-dependent decrease in the endogenous NFATc2 levels while silencing HDM2 prevented the Zoldronic acid (ZOL) -induced degradation of NFATc2. Further, it was demonstrated that the serine/kinase GSK-3β stabilized nuclear NFATc2 through the phosphorylation of the SP2 motif. This stabilization protected NFATc2 from HDM2-mediated proteolysis. Moreover, it was shown that in cancer cells, dephosphorylation of phospho-serines was a pre-requisite for ZOL-induced ubiquitination and proteasomal degradation of NFATc2. In contrast, constitutive phosphorylation of the SP2 motif to prevent NFATc2 degradation resulted in an increased cancer cell proliferation and these cells were significantly less responsive to ZOL-induced growth suppression (S. K. Singh et al. 2011).

Chao et al., 2019 showed that overexpression of carboxyl-terminus of Hsc70 interacting protein (CHIP), a U-box type chaperone associated E3- ubiquitin ligase, promotes the ubiquitination and degradation of NFATc3 through ubiquitin-proteasomal pathway. Further, their results suggest that CHIP decreases lipopolysaccharide (LPS)-induced cardiac hypertrophy and apoptosis in cardiomyoblasts possibly via proteasomal degradation of NFATc3 (Chao et al. 2019).

Yongna Fan et al., 2008 demonstrated that the ubiquitination of NFATc4 predominantly via Lys48 chains resulted in a decrease in the protein levels and transcriptional activity of NFATc4. Furthermore, the ubiquitination of NFATc4 was enhanced via activation of protein kinase GSK3β whereas the inhibition of GSK3β had opposite effects. Interestingly, the E3 ubiquitin-ligase that mediates the ubiquitination of NFATc4 had not been identified in this study (Y. Fan et al. 2008a).

Table XV : Summary of the plausible E3 ubiquitin-ligases identified and effect on the physiological function of different NFAT members. NA: Not applicable

<table>
<thead>
<tr>
<th>Substrate</th>
<th>E3 ubiquitin-ligase</th>
<th>Ubiquitination/degradation</th>
<th>Biological significance</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFATc1</td>
<td>Cbl-b and c-cbl</td>
<td>Yes</td>
<td>NA</td>
<td>(J. H. Kim et al. 2010; M.-Y. Youn et al. 2012; Xiaoxiao Li et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>VHL ubiquitin ligase associated with JMJD5; Cullin3 E3-ubiquitin ligase together with KBTBD</td>
<td>Yes</td>
<td>Negatively osteoclast differentiation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Decreases osteoclast differentiation</td>
<td></td>
</tr>
</tbody>
</table>

Although there are not many studies, it is clear that the regulation of NFAT by the UPS has a physiological impact. Interestingly, no E3 ubiquitin-ligase has been formally identified for NFAT except may be studies on HDM2, an E3 ubiquitin-ligase for NFATc2. Therefore, this is an important question which needs to be addressed.
<table>
<thead>
<tr>
<th>NFATc2</th>
<th>HDM2</th>
<th>Yes</th>
<th>Inhibition of breast cancer cell migration; cancer growth suppression (Yoeli-Lerner et al. 2005; S. K. Singh et al. 2011)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFATc3</td>
<td>CHIP</td>
<td>Yes</td>
<td>Induction of cardiac hypertrophy and apoptosis (Chao et al. 2019)</td>
</tr>
<tr>
<td>NFATc4</td>
<td>Not identified</td>
<td>Yes</td>
<td>NA (Y. Fan et al. 2008a)</td>
</tr>
</tbody>
</table>

**SUMOylation**

Studies have shown that SUMOylation plays a crucial role in the nuclear localization, transcriptional activity, and protein stability of NFAT proteins. Terui et al., 2004, showed that SUMOylation of NFATc2 on Lys 684 and Lys 897 is required for its transcriptional activity and nuclear anchorage, respectively. Interestingly, their results suggested that NFATc2 was recruited to SUMO-1 bodies and this recruitment might serve as a pre-requisite for the transcriptional activity of NFATc2 upon stimulation with ionomycin and phorbol12-myristate 13-acetate (PMA) (a stimulus which helps to induce the nuclear translocation of NFAT) (Figure 46a). Additionally, the homologous Lys 684 and K897 which fits the motif ψKXE is found in NFATc3 (Terui et al. 2004). Indeed, our group had demonstrated that NFATc3 contains three conserved SUMOylation sites (K435, K704, and K1013) and is conserved between mouse and human (Figure 46b). Notably, sites K704 and K1013 were important sites for SUMOylation and indeed SUMOylation prevented the nuclear translocation of NFATc3 through its interaction with Trim17 (Mojsa et al. 2015b).

Further, Nayak et al. 2009, demonstrated that the Lys residues 702 and 914 in the longer isoform of NFATc1, NFATc1/C, comprising of an extra C-terminal peptide of 246 amino acids were important sites for the SUMOylation. Moreover, upon SUMOylation, NFATc1/C translocated to PML nuclear bodies which led to interaction with histone deacetylases. This was followed by deacetylation of histones which induced transcriptionally inactive chromatin and as a result, suppressed IL-2, a target gene of NFATc1. Taken together, these results suggest that SUMO acts as a transcriptional repressor for NFATc1 (Nayak et al. 2009).

In a recent study, Kim et al., 2019 demonstrated that an isoform of NFATc1 namely NFATc1/A, composed of a short C-terminus and lacking the traditional SUMOylation sites found in the longer isoform, was modified by SUMO. In NFATc1/A, the lysine residue at 351 in the central regulatory domain was identified as the major SUMO attachment site and its SUMOylation increased with low expression levels of SUMO E3 ligases, PIAS1, PIAS3 and PIASγ. Interestingly, the SUMOylation of NFATc1/A did not affect its nuclear translocation, however, it slightly increased its protein stability (Figure 46a). Importantly, the transactivation activity of NFAT was negatively regulated by SUMOylation (E. T. Kim et al. 2019).
Altogether, these studies highlight the importance of SUMOylation in regulating NFAT family by controlling their protein stability, transcriptional activity, and sub-cellular localization. Figure 46 summarizes the impact of SUMO on different NFAT members.

Figure 46 (a) The experimentally validated SUMOylation sites in mouse NFATc1/A, human NFATc1/C, and mouse NFATc2/A are shown in the diagram. The different functional role of SUMOylation executed by different lysine residues on respective NFAT members are mentioned (E. T. Kim et al. 2019).

(b) Experimentally validated SUMOylation sites in NFATc3 (K435; K704; K1013) which are conserved between mouse and human.
Chapter 5  NFAT and neuronal Apoptosis

5.1 Apoptosis

*Introduction*

Multicellular organisms have a tightly regulated pathway of cellular development, maintenance, and removal. Apoptosis is a form of programmed cell death (PCD) (Kerr, Wyllie, and Currie 1972). It is characterized by a series of well-defined changes in morphology such as shrinkage of cells, chromatin condensation, membrane blebbing, and formation of apoptotic bodies, which are phagocytosed by neighbouring macrophages with no inflammatory response (Jayakiran 2015). It is a normal mechanism which is important for tissue homeostasis and development in all multicellular organisms (Fricker et al. 2018). Apoptosis is a critical process to maintain the balance between cell proliferation and elimination. Failure in the regulation of apoptosis can contribute to several diseases. For example, too little apoptosis can result in the uncontrolled growth and division of cells giving rise to cancer (Carneiro and El-Deiry 2020). In contrast, excess of apoptosis might be a contributing factor for neurodegenerative diseases such as Parkinson’s, Alzheimer’s (Dickson 2004), etc. Apoptosis results from the activation of a family of proteases known as caspases which are cysteine-aspartic proteases that cleave after aspartate residues and are themselves activated by proteolysis (two major pathways: extrinsic or death receptor pathway and intrinsic or mitochondrial pathway). Once activated, these proteases cleave key substrates and this cleavage induces some pathways which are responsible for death and the morphological characteristics of apoptosis (Van Opdenbosch and Lamkanfi 2019).

In this chapter, I would be describing briefly about apoptosis particularly neuronal apoptosis, and involvement of my protein of interest, NFATc3, and another redundant NFAT member, NFATc4, in neuronal apoptosis.

5.2 Pathways of Apoptosis

*Extrinsic pathway (or death receptor pathway)*

The extrinsic pathway is triggered by the activation of death receptors (DR) at the cell surface. DR are a class of receptors that belong to the TNFR superfamily and are characterized by the presence of death domains (DD). The binding of cognate ligands to the DR results in the recruitment of Fas-associated death domain protein (FADD) at the level of the intracellular part of the DR, which further binds to pro-caspase-8 to form the death-inducing signal complex (DISC). Several molecules of pro-caspase-8 are concentrated at the DISC and this concentration leads to auto activation of caspase 8 (Fricker et al. 2018). Consequently, the process induces both apoptotic and non-apoptotic pathways.
such as downstream cleavage of effector caspases-3 and -7 or IFN-β, TNF signaling (Fig. 31) (McArthur and Kile 2018).

**Intrinsic pathway (or Mitochondrial pathway)**

The intrinsic pathway or mitochondrial pathway is regulated by the mitochondria (MT) and BCL-2 family proteins at the level of MT. BCL-2 family can be grouped into: anti-apoptotic proteins (BCL-2, BCL-X, BCL-w, MCL-1, or BFL-1/A1), multi-domain pro-apoptotic proteins which reside in the MT (Bax and Bak), and BH3-only (BCL-2 homology 3) proteins (BID, BIM, BAD, BIK, BMF, Noxa, PUMA, and HRK).

When activated, the Bax and Bak pro-apoptotic proteins lead to a point of no return known as mitochondrial outer membrane permeabilization (MOMP) (M. X. Li and Dewson 2015; Tait and Green 2010; Galluzzi Lorenzo et al. 2012; Fricker et al. 2018). MOMP releases proteins that include cytochrome-c (Cyt-c) and the pro-apoptotic proteins Smac/DIABLO and Omi/HtrA2. Cyt-c release activates the formation of the apoptosome, a cytosolic multiprotein complex composed of Cyt-c, apoptotic protease activating factor 1 (Apaf-1), and Procaspase-9 (ProCASP9) (Gortat et al. 2015). This formation induces the activation of caspase-9 (Cavalcante et al. 2019). Interestingly, Smac and Omi interact with inhibitory proteins to activate procaspases such as ProCaspase-3 (Pro-C3) and ProCaspase-7 (ProC7) (T. C. Cheng et al. 2016). On the other hand, BH3-only proteins are pro-apoptotic proteins that can either inhibit anti-apoptotic proteins or activate pro-apoptotic proteins resulting in the activation of caspases (Lomonosova and Chinnadurai 2008).
The initiator CASP9 cleaves and activates the execution caspases such as Caspase-3 (CASP-3) and Caspase-7 (CASP-7) via proteolysis which rearranges important protein loops in the formation of active sites (Shi 2004). When activated, these execution caspases can cleave and activate other execution caspases during the execution phase (S. Yuan et al. 2011) Figure 47 (McArthur and Kile 2018).

**Execution phase**

The execution phase of apoptosis is the final part of the cell death process. It proceeds when the cell is fully committed to die and there is a rapid occurrence of cell death after the initiation of this phase. Caspases play a crucial role in this phase and are responsible for the morphological characteristics of apoptosis by cleaving key substrates. They are classified as initiators such as CASP2, CASP8, CASP9, and CASP10 or effectors/executioners such as CASP3, CASP6, and CASP7 (Shalini et al. 2015; Enari et al. 1998). The initiator caspases activate the executioner caspases, which in turn activate other executioners in a feedback loop that affects essential structural proteins and other enzymes in order for the key features of apoptotic cell death to occur (McIlwain, Berger, and Mak 2013). Finally, executioner caspases activate the cytoplasmic endonucleases which degrade nuclear material and proteases which degrade core and cytoskeletal proteins (Elmore 2007b).

### 5.3 Neuronal apoptosis

**Physiological and pathophysiological implications**

During embryogenesis, apoptosis plays a major role in the development of nervous system as neuronal precursors differentiate to become post-mitotic neurons (Hollville, Romero, and Deshmukh 2019). Interestingly, during normal nervous system development, physiologically appropriate neuronal loss contributes to a sculpting process that eliminates approximately 50% of all neurons born during neurogenesis. Indeed, only the neurons which have established correct synaptic connections with their targets or other afferent neurons would be able to obtain the sufficient level of neurotrophic factors and excitatory stimulation to survive (Yamaguchi and Miura 2015).

After the incorporation of neurons into functional circuits and maturation of neurons, the capacity of neurons to die via apoptosis is greatly restricted. This allows the mature nervous system to persist in a healthy and functional state throughout life. Interestingly, in the context of pathology, these mature neurons are still capable of re-activating the apoptotic pathway. This could contribute to neurodegenerative disorders that include Alzheimer’s disease and Parkinson’s disease (Hollville, Romero, and Deshmukh 2019). Therefore, illustrating the molecular mechanisms underlying neuronal apoptosis might contribute to understanding the basis of human neuropathology. Further, this could potentially aid in identifying specific treatments and preventive strategies in the context of disease.
Cerebellar granule neurons (CGNs) as a model for induction of apoptosis

My group has been using primary cultures of CGNs as a model to study neuronal apoptosis (Irina Lassot et al. 2010b; Magiera et al. 2013a; Mojsa et al. 2015a). These neurons survive and differentiate in vitro in the presence of serum and 25mM of KCl. This high level of KCl induces a depolarization that mimics the excitation by afferent neurons that is required for survival of CGNs in vivo. After 6 days in vitro, removal of serum and a low concentration of KCl to 5mM triggers apoptosis in neurons (D’Mello et al. 1993). This recapitulates the apoptotic process that occurs in vivo during post-natal development. Interestingly, apoptosis is regulated at the transcriptional level in neurons (Ham et al. 2000). Notably, in CGNs, neuronal death can be prevented with the use of transcription inhibitors and many transcription factors controlling neuronal apoptosis have been identified. Moreover, the NFAT family particularly NFATc3 and NFATc4, play a crucial role in the development of the nervous system and in the control of the survival/death fate of neurons (Mojsa et al. 2015b; Gómez-Sintes and Lucas 2010; Jayanthi et al. 2005; Benedito et al. 2005; Vashishta et al. 2009; Quadrato et al. 2012). Additionally, numerous studies suggest UPS as a regulator of neuronal apoptosis and proteasome inhibition prevents CGN apoptosis (Magiera et al. 2013a; Irina Lassot et al. 2010b; Butts et al. 2005). Therefore, CGNs constitute an ideal model to study protein degradation regulating apoptosis (Figure 48).

![Figure 48](image)

Figure 48: CGNs deprived of serum and KCl as a model of transcription-dependent apoptosis. Phase contrast and electron microscopy images of control (FBS+25mM KCl) or apoptotic CGN (-FBS+5mM KCl, 16h of treatment) after 6 days of in vitro culture (DIV 6).

5.4 NFATc3 and NFATc4- similar but different too

**Expression**

High levels of NFATc3 and NFATc4 transcripts were found in the cerebellum, substantia nigra (SN), cerebral cortex, striatum, and corpus callosum in the brain. Interestingly, high levels of NFATc4 was
observed at E13 stage during brain development and a decrease in its expression was observed thereafter with the lowest levels observed at postnatal day 14 (Vihma, Pruunsild, and Timmusk 2008; Jayanthi et al. 2005). Interestingly, NFATc3 has been found in the hypothalamic growth-hormone releasing-hormone (GHRH) neurons where it is involved in the depolarization-induced transcriptional activation of GHRH gene in the neuronal cells (Asai et al. 2004). NFATc3 and NFATc4 was found to be expressed in hippocampal neurons, cortical neurons, striatal neurons, and cerebellar granule neurons (Abdul et al. 2009; Vihma et al. 2016b; Benedito et al. 2005; Mojsa et al. 2015a; Vashishta et al. 2009; Jayanthi et al. 2005; Groth et al. 2008). Strikingly, we had observed that mRNA levels of NFATc3 was 10 times higher than that of NFATc4 in the CGNs (Mojsa et al. 2015a). Importantly, it has been reported that NFATc3 and c4 are the most predominant NFAT genes in dorsal root ganglion (DRG) neurons (M.-S. Kim and Usachev 2009). It should be noted that apart from NFATc3 and -c4 other NFAT members: NFAT c1, and -c2 are also expressed in neurons (A. M. Ho et al. 1994; Vihma et al. 2016b).

Subcellular localization and intracellular kinetics

The regulation of subcellular localization and the transcriptional activity of NFAT family members in neurons depends on the specific genes. The regulation of NFATc3 and NFATc4 nuclear translocation has been studied by various groups. The activation of L-type calcium channels by high levels of extracellular K+ results in the nuclear translocation of NFATc3 and NFATc4 in hippocampal and dorsal root ganglion neurons (I. A. Graef et al. 1999; Oliveria, Dell’Acqua, and Sather 2007; Ulrich et al. 2012; Murphy et al. 2014). Interestingly, brain-derived neurotrophic factor application and D1 dopamine receptor signaling has been shown to trigger a rapid nuclear translocation of NFATc4 in hippocampal neurons (Groth and Mermelstein 2003), in cortical neurons (Isabella A. Graef et al. 2003), and striatal neurons (Groth et al. 2008), respectively. Additionally, it was also found that treatment of N-methyl-D-aspartate (NMDA-a glutamate receptor and ion channel protein found in nerve cells) induces the nuclear translocation of both NFATc3 and NFATc4 in cortical neurons (Vashishta et al. 2009). Further, in an interesting study carried out by Luo et al., 2014, it was demonstrated that in a transgenic mouse model of Parkinson’s disease, an increase in the cytosolic calcium concentration and CaN activity led to a significant increase in the nuclear distribution of NFATc3 in midbrain dopaminergic neurons (J. Luo et al. 2014). On the other hand, Caraveo et al., 2014, showed an increase in the nuclear staining of NFATc4 in human α-synucleinopathy patients’ brains compared to the healthy controls (Caraveo et al. 2014). Finally, Mojsa et al., 2015, demonstrated that Trim17 favored the cytoplasmic localization of NFATc3 and NFATc4, therefore, inhibiting their nuclear translocation (Mojsa et al. 2015a).

Interestingly, NFATc3 and NFATc4 differ in their translocation kinetics. Ulrich et al., 2012, had demonstrated that NFATc3 underwent a rapid dephosphorylation and nuclear translocation (complete in 20 minutes), however, NFATc4 remained phosphorylated, localized to the cytosol, and displayed nuclear translocation only after prolonged (1-3h) depolarization. Furthermore, only the knockdown of NFATc3 but not NFATc4 strongly diminished transcription mediated by NFAT upon induction by mild depolarization in neurons. The authors also reasoned that this delay in depolarisation-induced nuclear translocation of NFATc4 might be due to the inhibition of NFATc4
by GSK3β (NFAT maintenance kinase). Therefore, NFATc3 is strongly activated in neurons while
the activation of NFATc4 requires both an increase in Ca\textsuperscript{2+} and GSK3β suppression which gives rise
to the distinct activation of these two NFAT members (Ulrich et al. 2012).

**Function of NFATc3 and NFATc4 in neuronal apoptosis**

Over the years, NFAT family members have established themselves as regulators of transcription
and NFAT-dependent gene regulation has been implicated in diverse cellular processes such as
survival, apoptosis, differentiation, and proliferation.

The nuclear translocation of NFAT holds the key for the transcription of its downstream target
genes. Vihma et al., 2016 tested the transactivation capacities of over-expressed human NFAT
isoforms in rat primary cortical and hippocampal neurons and in HEK293 cells. They found that
amongst different NFAT members, NFATc3 and NFATc4 are the strongest transcriptional activators
and lead to the highest induction in neurons (Vihma et al. 2016b).

NFATc3 and NFATc4 coordinate the expression of many genes which have a direct effect on
neuronal apoptosis. For example, Mojisa et al., 2015 demonstrated that Brain derived nerve growth
factor (Bdnf) is a target gene of both NFATc3 and NFATc4 whereas the pro-apoptotic gene Trim17
is a target gene of NFATc3 alone. Interestingly, she had shown in this study that NFATc3 induced
the transcription of Trim17 in association with c-Jun.

Depending on the physiologic and cellular context, both NFATc3 and NFATc4 have been shown to
be either proapoptotic or antiapoptotic. Jayanthi et al., 2005 had observed that Methamphetamine
(Meth), an abused psychostimulant, increased calcineurin levels which in turn increased the nuclear
translocation of both NFATc3 and NFATc4. This resulted in an increase in FasL-Fas death pathway
and hence, in an increase in neuronal apoptosis (Jayanthi et al. 2005). Further, Gomez-Sintes et al.,
2010 had demonstrated that the inhibition of GSK3β by lithium (used in the treatment of bipolar
disorder, Alzheimer’s disease and other neurodegenerative disorder) led to an increase in the nuclear
translocation of NFATc3, thereby increasing the levels of FasL and Fas activation. The results of this
study highlighted the mechanism for lithium-induced neuronal and motor toxicity (Gómez-Sintes
and Lucas 2010).

Indeed, in the previous study, my group had shown that NFATc3 and NFATc4 have opposite effects
on neuronal apoptosis (Mojsa et al. 2015a). Data from other studies suggested that NFATc4 sustained
survival in different types of neurons by inducing the transcription of survival factors (Benedito et
al. 2005; Vashishta et al. 2009; Quadrato et al. 2012). In line with observations of others, our group
had shown that the transfection of NFATc4 protected CGNs from apoptosis. Moreover, we also
showed that silencing of NFATc3 protected CGNs from serum/KCl deprivation whereas
overexpression of NFATc3 aggravated apoptosis in CGNs. Therefore, NFATc3 favors neuronal
apoptosis while NFATc4 protects neurons from survival factor withdrawal induced apoptosis (Mojisa
et al. 2015a).
Objectives

My group has been working on Trim17 for 15 years. They had identified Trim17 as a crucial E3 ubiquitin ligase that is necessary and sufficient for neuronal apoptosis (Irina Lassot et al. 2010a; Magiera et al. 2013a). During her PhD, a former student, Barbara Mojsa, had shown that Trim17 could interact with both NFATc3 and NFATc4 in neuronal cells. Strikingly, she found that Trim17 slightly decreased the ubiquitination level [Figure 49(b)] and increased the protein levels of NFATc3 [Figure 49(a)]. Therefore, it was clear that Trim17 is not an E3 ubiquitin-ligase for NFATc3 (Mojsa et al. 2015a). NFAT are relatively short-lived proteins and regulating their stability is important for controlling their activity. Further, as suggested by few studies, regulation of NFATs by the UPS can have an impact on their physiological function (S. K. Singh et al. 2011; Xiaoxiao Li et al. 2015, 77; Narahara et al. 2019). As mentioned before, of all the NFAT members, NFATc3 is the predominant NFAT gene expressed in neurons (Ulrich et al. 2012). Notably, NFATc3 has a high level of expression in CGNs (Mojsa et al. 2015a). Furthermore, NFATc3 was found to be strongly activated in neurons (Ulrich et al. 2012) and to favor neuronal apoptosis (Caraveo et al. 2014; Jayanthi et al. 2005; Gómez-Sintes and Lucas 2010; Mojsa et al. 2015a). Strikingly, the regulation of its protein level has not been studied and no genuine E3 ubiquitin-ligase of NFATc3 has been identified so far.

Further, TRIM family members are known to interact with each other. In parallel studies, the group has demonstrated that TRIM17 can interact with other TRIM proteins, TRIM28 and TRIM41, and inhibit their E3 ubiquitin-ligase activity on their substrates (BCL2A1 and ZCAN21, respectively).

Figure 49: Trim17 is not an E3 ubiquitin-ligase of NFATc3 (Mojsa et al. 2015a)
(a) shows an increase at the protein level of NFATc3 with increasing amounts of Trim17.
(b) shows a decrease in the ubiquitination of NFATc3 with increasing amounts of Trim17.
(c) shows a decrease in the ubiquitination of NFATc3 with increasing amounts of Trim17.
Therefore, the stability of NFATc3 might be regulated by a TRIM protein interacting with Trim17. Three systematic yeast two-hybrid screens had shown that human TRIM39 and TRIM17 proteins interact with each other (Rual et al. 2005; Woodsmith, Jenn, and Sanderson 2012; Rolland et al. 2014). Barbara Mojsa indeed confirmed that mouse Trim17 and Trim39 interact with each other. She also tested the effect of other partners of Trim17 (such as Trim41 and Trim44) on the protein level of NFATc3 and found that the protein level of NFATc3 was decreased only in the presence of Trim39 (Figure 50). She further found that indeed Trim39 strongly reduced the ubiquitination of NFATc3 in a dose-dependent manner, however, the negative controls were missing in these experiments. In contrast, she also showed that silencing endogenous Trim39 abrogated the ubiquitination of NFATc3. Some of her unpublished results suggest that NFATc4 is also a substrate of Trim39. Following the observation of these results and in line with other studies in the group, we hypothesized that Trim17 might exert an inhibitory effect also on Trim39-mediated ubiquitination of NFATc3.

Moreover, the previous experiments conducted by Barbara Mojsa suggested the importance of SUMOylation of NFATc3. Notably, it was shown that K704 and K1013 of NFATc3 were important sites of SUMOylation. Additionally, Barbara found that the NFATc3-KallR mutant (where all the three Lys residues: K435, K704, K1013 were mutated to Arg) was more stable than the WT-NFATc3. However, these K residues could either be ubiquitinated or SUMOylated. Therefore, to differentiate the effects of SUMOylation and ubiquitination on NFATc3, she generated the EA mutant of NFATc3 where the second conserved Glu (E) residue in the SUMOylation consensus motif was mutated to Ala (A). Interestingly, she showed that the NFATc3-EallA mutant was less ubiquitinated by Trim39 than the WT-NFATc3 which raised many questions regarding the crosstalk between ubiquitination and SUMOylation on NFATc3. The preliminary results of Barbara Mojsa were indeed very promising and owing to time constraints, this study could not be developed further by her.
Therefore, during my PhD, based on the preliminary results, I have addressed the following questions (Figure 51):

1. Is Trim39 a genuine E3 ubiquitin-ligase of NFATc3?
2. Does Trim17 inhibit Trim39-mediated ubiquitination of NFATc3 and if so, what are the mechanisms involved?
3. What is the role of SUMOylation in the ubiquitination and stability of NFATc3?
4. Is Trim39 a SUMO-targeted E3 ubiquitin-ligase for NFATc3?
5. Does SUMOylation and Trim39 modulate the physiological function of NFATc3?

Figure 51: Schematic representation of the questions raised on the regulation of NFATc3.
Results

Article

**Trim39 regulates neuronal apoptosis by acting as a SUMO-targeted E3 ubiquitin-ligase for the transcription factor NFATc3**

M Basu Shrivastava, B Mojsa, S Mora, I Robbins, G Bossis, I Lassot, S Desagher*

**Introduction**

In a previous study, my group had shown that Trim17 binds to NFATc3 and NFATc4 and inhibits their nuclear-translocation (Mojsa et al. 2015b). Further, the SUMOylation sites of NFATc3 have been shown to be important for its interaction with Trim17. Importantly, it was also shown that NFATc3 induces neuronal apoptosis, at least in part, by increasing the expression of the pro-apoptotic protein Trim17. Although, NFATc3 is the predominant NFAT gene expressed in neurons and has been shown to play an important role in neuronal apoptosis, the regulation of NFATc3 stability has been poorly studied. Strikingly, no genuine E3 ubiquitin-ligase of NFATc3 has been identified so far.

When I arrived in the group, the former PhD student Barbara Mojsa, already had preliminary results suggesting that Trim39 was an E3 ubiquitin-ligase of NFATc3. Interestingly, she also observed that SUMOylation sites were important for the ubiquitination of NFATc3. All these results were very promising and additional experiments were needed to understand the mechanisms regulating the stability of NFATc3. Initially, the goal was to finish the experiments to demonstrate that Trim39 is an E3 ubiquitin-ligase of NFATc3. For this, I performed experiments such as in-vivo ubiquitination, co-immunoprecipitation, half-life measurement, effect of Trim39 on the protein level, and on the NFATc3 target genes. However, during my PhD, I had great opportunities to attend different conferences and present my results. Recurring questions being asked on whether Trim39 has SIMs, motivated me to go deep into this study and examine whether Trim39 could be a STUbL of NFATc3. This was really exciting as only few STUbLs are known in the field. With the help of Guillaume Bossis’s discussions, methodology, and material support, my results strongly suggest that Trim39 is indeed a STUbL of NFATc3. For this, I performed different experiments such as site-directed mutagenesis to make different SIM mutants, binding of SUMO-2 chains, co-immunoprecipitation and in-vivo ubiquitination. I further confirmed the binding of Trim39 to 4xSUMO (material kindly provided by Manuel Rodriguez-unpublished results). Furthermore, Stephan Mora confirmed the interactions between endogenous NFATc3 and Trim39, and inhibition of this interaction by Trim17 by proximity ligation assay. He also performed the experiments to address the physiological relevance of SUMOylation and Trim39 on NFATc3 using CGN as a model. Finally, the in-vitro ubiquitination and in-vitro SUMOylation experiments were done by Irena Lassot.
Altogether, in the present study, we have shown that Trim39 is an E3 ubiquitin-ligase of NFATc3 and that Trim17 inhibits the Trim39-mediated ubiquitination of NFATc3. Moreover, our results indicate that SUMOylation sites are important for the stability and ubiquitination of NFATc3 by Trim39 strongly suggesting that Trim39 is a STUbL of NFATc3. Finally, we also show that the transcriptional activity of NFATc3 and its pro-apoptotic effect in neurons are decreased by both SUMO and Trim39.
Trim39 regulates neuronal apoptosis by acting as a SUMO-targeted E3 ubiquitin-ligase for the transcription factor NFATc3

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Abstract (150 words)

NFATc3 is the predominant member of the NFAT family of transcription factor in neurons, where it plays a pro-apoptotic role. Mechanisms controlling NFAT protein stability are poorly understood. Here we identify Trim39 as an E3 ubiquitin-ligase of NFATc3. Indeed, Trim39 ubiquitinates NFATc3 in vitro and in cells, whereas silencing of endogenous Trim39 decreases NFATc3 ubiquitination. We also show that Trim17 inhibits Trim39-mediated ubiquitination of NFATc3 by reducing both the E3 ubiquitin-ligase activity of Trim39 and the NFATc3/Trim39 interaction. Moreover, mutation of SUMOylation sites in NFATc3 or SUMO-interacting motif in Trim39 reduces the NFATc3/Trim39 interaction and Trim39-induced ubiquitination of NFATc3. As a consequence, silencing of Trim39 increases the protein level and transcriptional activity of NFATc3, resulting in enhanced neuronal apoptosis. Likewise, a SUMOylation-deficient mutant of NFATc3 exhibits increased stability and pro-apoptotic activity. Taken together, these data indicate that Trim39 modulates neuronal apoptosis by acting as a SUMO-targeted E3 ubiquitin-ligase for NFATc3.
Introduction

The NFAT (Nuclear Factor of Activated T cells) family of transcription factors is a key player in a wide range of physiological and pathological processes. Initially discovered in activated T cells (Shaw et al., 1988), the different members of the NFAT family have been identified in most tissues where they play both redundant and specific roles (Fric et al., 2012; Kipanyula et al., 2016; Mognol et al., 2016; Wu et al., 2007). They are implicated in the development and the function of the immune system, brain, cardiovascular system, skeletal muscles, bones and other organs by regulating the expression of different target genes involved in cytokine production but also in cell proliferation, differentiation and apoptosis. As a consequence, NFAT deregulation is involved in many pathologies including auto-immune diseases, cancer and neurodegenerative diseases (Kipanyula et al., 2016; J.-U. Lee et al., 2018; Müller & Rao, 2010). A better understanding of NFAT regulation, in particular by post-translational modification and degradation, is therefore of crucial importance.

The calcium-regulated, cytoplasmic-nuclear shuttling of NFATc1, NFATc2, NFATc3 and NFATc4 has been extensively studied. These NFAT members are normally found in the cytoplasm in a hyperphosphorylated and inactive state. Upon an increase in intracellular calcium levels, they are dephosphorylated by the calcium-dependent phosphatase calcineurin, which triggers their nuclear import and activation. Once in the nucleus, NFATs induce (or repress) the transcription of specific target genes, usually in cooperation with partner transcription factors such as AP-1 or co-activators (Hogan et al., 2003; Mognol et al., 2016; Müller & Rao, 2010). In contrast, the regulation of NFAT stability by the ubiquitin-proteasome system remains elusive. Indeed, only a few studies have addressed this issue. However, NFATs are relatively short-lived proteins and previous studies have shown that interfering with the regulation of NFAT levels by the ubiquitin-proteasome system can have a marked impact on the physiology of various cell types (Chao et al., 2019; X. Li et al., 2015; Narahara et al., 2019; Singh et al., 2011; Yoeli-Lerner et al., 2005; Youn et al., 2012). In addition to phosphorylation and ubiquitination, NFAT proteins have been shown to be regulated by SUMOylation. Several studies have shown that covalent conjugation of SUMO to NFATs has an impact on their cytoplasmic-nuclear shuttling, subnuclear localization and transcriptional activity (E. T. Kim et al., 2019; Nayak et al., 2009; Terui et al., 2004; Vihma & Timmusk, 2017). Indeed, SUMOylation can have many consequences on its substrate proteins, including modification of their activity, interaction properties and subcellular localization (Henley et al., 2018; X. Zhao, 2018). In addition, SUMOylation of proteins can regulate their stability (Liebelt & Vertegaal,
2016). Indeed, a few E3 ubiquitin-ligases that specifically recognize and ubiquitinate SUMOylated proteins have been described (Geoffroy & Hay, 2009; Prudden et al., 2007; Sriramachandran & Dohmen, 2014). These SUMO-targeted E3 ubiquitin-ligases (STUbLs) generally induce the degradation of their substrates by the proteasome, raising the possibility that SUMO might also modulate NFAT ubiquitination and degradation.

NFATc3 is the predominant NFAT family member expressed in various neuronal types (M. S. Kim & Usachev, 2009; Luo et al., 2014; Mojsa et al., 2015; Ulrich et al., 2012; Vashishta et al., 2009). We have previously shown that NFATc3 is involved in the regulation of neuronal apoptosis (Mojsa et al., 2015). Two independent studies have also implicated NFATc3 in α-synuclein-induced degeneration of midbrain dopaminergic neurons in Parkinson’s disease (Caraveo et al., 2014; Luo et al., 2014). Interestingly, following depolarization-induced elevations of intracellular calcium concentration in neurons, NFATc3 is more rapidly and strongly activated than NFATc4, (Ulrich et al., 2012). Once in the nucleus, activation of pro-apoptotic protein kinases such as GSK3β does not seem to be sufficient to induce NFATc3 nuclear exclusion in neurons (Mojsa et al., 2015; Ulrich et al., 2012). Proteasomal degradation could therefore be an alternative way to reduce its activity in this case. However, only one study relating NFATc3 ubiquitination and degradation has been reported so far and this in the context of LPS-induced cardiac hypertrophy (Chao et al., 2019). In previous work, we have shown that NFATc3 can be SUMOylated on three consensus sites (Mojsa et al., 2015). We have also found that NFATc3 binds to Trim17 (Mojsa et al., 2015), which belongs to a large family of RING-containing E3 ubiquitin-ligases. Although its E3 ubiquitin-ligase activity has been confirmed (I. Lassot et al., 2010; Urano et al., 2009), Trim17 does not induce NFATc3 ubiquitination. On the contrary, overexpression of Trim17 reduces the ubiquitination of NFATc3 and increases its steady-state protein level (Mojsa et al., 2015). Since TRIM17 can prevent ubiquitination of some of its binding partners by inhibiting other E3 ubiquitin-ligases from the TRIM family (Iréna Lassot et al., 2018; Lionnard et al., 2019), we hypothesized that the stability of NFATc3 might be regulated by a TRIM protein interacting with Trim17, such as Trim39.

In the present study, we demonstrate that Trim39 is a genuine E3 ubiquitin-ligase for NFATc3. We also show that Trim39-mediated ubiquitination of NFATc3 is inhibited by Trim17. Moreover, mutation of NFATc3 SUMOylation sites both decreases its ubiquitination by Trim39 and increases its stability. The same effects are reproduced by mutation of a crucial SUMO-interacting motif (SIM) in Trim39. These data indicate that Trim39 acts as a STUbL for NFATc3. As a result, SUMO and Trim39 modulate the transcriptional activity of NFATc3.
and its pro-apoptotic effect in neurons. Therefore, our study provides the identification of a new STUbL and a first insight into complex mechanisms regulating the stability of NFATc3 in neurons.

Results

 Trim39 is an E3 ubiquitin-ligase for NFATc3

Human TRIM39 and TRIM17 proteins have been found to interact with each other in three independent proteome-scale yeast two-hybrid screens (Rolland et al., 2014; Rual et al., 2005; Woodsmith et al., 2012). To determine whether mouse Trim39 and Trim17 proteins can also bind to each other, and whether Trim39 can bind to NFTAc3, co-immunoprecipitation experiments were performed. Indeed, in cells co-transfected with Trim17-GFP and Flag-Trim39, immunoprecipitation of Trim39 using anti-Flag antibody co-precipitated Trim17, whereas immunoprecipitation of Trim17 using GFP-Trap beads co-precipitated Trim39 (Fig. 1A). In a similar way, in cells co-transfected with HA-NFATc3 and Flag-Trim39, the two proteins were reciprocally co-immunoprecipitated by using either anti-Flag or anti-HA antibodies (Fig. 1B). To confirm this interaction at the endogenous level, we next performed an in situ proximity ligation assay (PLA) in Neuro2A cells, using anti-NFATc3 and anti-Trim39 antibodies. Close proximity was detected between endogenous NFATc3 and endogenous Trim39 as assessed by a PLA signal (Fig. 1C) that was predominantly cytoplasmic (Fig. 1C, endo 1 slice). Overexpression of Trim39 increased the PLA signal (Fig. 1C). To confirm the specificity of the assay, we used a specific shRNA against Trim39 and first verified that it effectively reduces the level of endogenous Trim39 protein (Fig. 1D). As expected, silencing of Trim39 using this shRNA strongly decreased the PLA signal (Fig. 1E). Taken together, these data indicate that Trim39 interacts with both Trim17 and NFATc3.

We next examined whether Trim39 could mediate the ubiquitination of NFATc3. In cells co-transfected with His-tagged ubiquitin, the ubiquitination level of NFATc3 was significantly increased by overexpressed Trim39 but not by an inactive mutant deleted of its RING domain (Trim39-ΔRING; Fig. 2A). In contrast, silencing of Trim39 using three different specific shRNAs, deeply decreased the ubiquitination of NFATc3 (Fig. 2B), indicating that endogenous Trim39 is involved in the ubiquitination of NFATc3. To further demonstrate that Trim39 is an E3 ubiquitin-ligase of NFATc3, we carried out an in vitro ubiquitination assay using in vitro translated/immuno-purified NFATc3 and purified recombinant proteins. In these experiments,
GST-Trim39 stimulated NFATc3 ubiquitination in the presence of ubiquitin, E1 and E2 enzymes but not in the absence of ubiquitin (Fig. 2C). In contrast, an inactive mutant of Trim39 in which two crucial Cys residues of the RING domain where mutated (GST-Trim39-C49S/C52S) did not have any effect (Fig. 2C). Taken together, these data indicate that NFATc3 is a direct substrate for the E3 ubiquitin-ligase activity of Trim39.

**Trim39 induces the degradation of NFATc3 and decreases its transcriptional activity**

Because ubiquitination often targets proteins for proteasomal degradation, we examined whether Trim39 could impact the protein level of NFATc3. Indeed, the level of NFATc3 progressively decreased when co-transfected with increasing amounts of Trim39 (Fig. 3A). Interestingly, the inactive mutant Trim39-ΔRING did not decrease the protein level of NFATc3 but rather increased it, in a similar way as the proteasome inhibitor MG-132 (Fig. 3A). Mutations of the RING domain of E3 ubiquitin-ligases generally induce a dominant-negative effect (I. Lassot et al., 2010; Pickart, 2001). Therefore, this increase in NFATc3 protein may be due to the inhibition of endogenous Trim39 by Trim39-ΔRING, as it has been previously reported for the effect of Trim39 on the half-life of p53 (Zhang, Huang, et al., 2012). Consistently, silencing of endogenous Trim39 using a specific siRNA also significantly increased the protein level of endogenous NFATc3 in Neuro2A cells (Fig. 3B). Taken together, these data strongly suggest that Trim39-mediated ubiquitination is involved in the proteasomal degradation of NFATc3.

To examine whether the effect of Trim39 on the protein level of NFATc3 could have an impact on its activity as a transcription factor, we measured the mRNA level of one of its target genes: *Trim17*. Indeed, in a previous study, we have shown that Trim17 is transcriptionally induced by NFATc3 (Mojsa et al., 2015). Consistently, in the present study, Trim17 mRNA level was increased when NFATc3 was overexpressed (Fig. 3C). Interestingly, this induction was completely abrogated by co-expression of wild type but not inactive Trim39 (ΔRING). Moreover, even when NFATc3 was not transfected, Trim39-ΔRING significantly increased the expression level of Trim17 (Fig. 3C), suggesting that the inhibition of endogenous Trim39 through a dominant negative effect is sufficient to increase the activity of endogenous NFATc3 (Fig. 3A). To confirm these data, Neuro2A cells were treated with the calcium ionophore A23187 and phorbol 12-myristate 13-acetate (PMA) to activate both endogenous NFATc3 (through calcium-induced nuclear translocation) and its transcriptional partner AP-1. As
previously reported (Mojsa et al., 2015), Trim17 mRNA level was increased following treatment with A23187 and PMA (Fig. 3D). Although increase in intracellular calcium should activate other members of the NFAT family, this induction of Trim17 is probably due to NFATc3 as it is the NFAT transcription factor that is predominantly expressed in Neuro2A cells (Mojsa et al., 2015). Again, this Trim17 induction was completely abrogated by overexpression of wild type Trim39 but not inactive Trim39 (Fig. 3D). Overexpression of the dominant-negative mutant Trim39-ΔRING also significantly increased the expression level of Trim17, even in control conditions (Fig. 3D). Taken together, these data suggest that exogenous Trim39 reduces the transcriptional activity of both overexpressed and endogenous NFATc3. To determine the impact of endogenous Trim39, Neuro2A cells were transfected with two different specific siRNAs that efficiently decreased the mRNA level of Trim39 (Fig. 3E, left panel). Silencing of Trim39 resulted in Trim17 induction, notably following treatment with A23187 and PMA, which activates endogenous NFATc3 (Fig. 3E, right panel). These data therefore indicate that endogenous Trim39 also regulates endogenous NFATc3. As we have previously shown that Trim17 can bind and inhibit NFATc3 by preventing its nuclear translocation (Mojsa et al., 2015), we examined whether Trim39 could have the same effect on NFATc3. Indeed, under conditions where Trim17 decreased the nuclear translocation of NFATc3 by more than twofold, Trim39 had no impact on the subcellular localization of NFATc3 (Fig. S1). Therefore, these data strongly suggest that Trim39 inhibits the transcription factor activity of NFATc3 by ubiquitinating it and by inducing its proteasomal degradation, but not by preventing its nuclear translocation.

Trim17 inhibits the ubiquitination of NFATc3 mediated by Trim39

As we initially observed that Trim17 decreases the ubiquitination level of NFATc3 (Mojsa et al., 2015), we tested whether Trim17 could affect Trim39-mediated ubiquitination of NFATc3. Indeed, the increase in NFATc3 ubiquitination induced by Trim39 overexpression was abolished by the co-expression of Trim17 in cells (Fig. 4A). This effect was confirmed in vitro. Indeed, the ubiquitination of in vitro translated NFATc3 by recombinant His-TRIM39 was completely prevented by recombinant MBP-TRIM17 (Fig. 4B). As only purified proteins were used in a complete acellular medium for this assay, these results suggest that TRIM17 can directly inhibit the ubiquitination of NFATc3 induced by TRIM39. Interestingly, the ubiquitination level of Trim39 was strongly decreased in the presence of Trim17 both in cells (Fig. 4A) and in vitro (Fig. 4B), excluding the possibility that Trim17 acts by ubiquitinating
Trim39. Moreover, as in vitro auto-ubiquitination gives a measure of E3 ubiquitin-ligase activity (Pickart, 2001), this also suggests that TRIM17 can directly inhibit the E3 ubiquitin-ligase activity of TRIM39. Interestingly, in these experiments, the in vitro auto-ubiquitination of TRIM17 was also decreased (Fig. 4B) by TRIM39 and the ubiquitination level of Trim17 in cells was also reduced in the presence of Trim39 (Fig. S2), suggesting a reciprocal inhibition of the two TRIM proteins.

To further investigate the mechanisms underlying the inhibitory effect of Trim17, the impact of Trim17 on the interaction between NFATc3 and Trim39 was assessed. Strikingly, when co-transfected with HA-NFATc3 and Flag-Trim39, Trim17-GFP almost completely prevented the co-immunoprecipitation of Flag-Trim39 with HA-NFATc3 (Fig. 5A) or the co-immunoprecipitation of HA-NFATc3 with Flag-Trim39 (Fig. 5B). Moreover, in PLA experiments, the close proximity signal between endogenous NFATc3 and Trim39 proteins was significantly reduced by the overexpression of Trim17-GFP compared to GFP (Fig. 5C,D).

Taken together, these data strongly suggest that Trim17 inhibits the ubiquitination of NFATc3 mediated by Trim39 by inhibiting both the intrinsic E3 ubiquitin-ligase activity of Trim39 and the interaction between NFATc3 and Trim39.

SUMOylation of NFATc3 modulates its ubiquitination and stability

In a previous study, we have identified three consensus SUMOylation sites in NFATc3 (Mojsa et al., 2015). As SUMOylation can modify the stability of proteins (Liebelt & Vertegaal, 2016), we tested whether alteration of the SUMOylation of NFATc3 can have an impact on its ubiquitination and half-life. We had previously used NFATc3 K/R mutants in which the acceptor Lys residues of the SUMOylation consensus motifs were replaced by Arg (Mojsa et al., 2015). However, large-scale mass spectrometry studies have shown that a quarter of SUMO acceptors lysines are also used for ubiquitin modification (Liebelt & Vertegaal, 2016). Therefore, additional NFATc3 mutants were generated in order to prevent SUMOylation without affecting a possible ubiquitination at these sites. For this purpose, the Glu residues of the NFATc3 SUMOylation consensus motifs (ψKXE with ψ representing a large hydrophobic residue and X any amino acid (Pichler et al., 2017; Rodriguez et al., 2001)) were substituted for Ala to generate NFATc3 E/A mutants. As expected, in vitro SUMOylation of the NFATc3-EallA mutant (in which the Glu residues of the three consensus motifs were replaced by Ala) and the NFATc3-KallR mutant (in which the Lys residues of the three consensus motifs were
replaced by Arg), was almost completely abrogated (Fig. 6A). Interestingly, the ubiquitination level of NFATc3 in Neuro2A cells was not significantly altered by single or double E/A mutations whereas it was strongly decreased by the triple mutation (Fig. 6B), suggesting that SUMOylation of at least one consensus motif is necessary to favour the ubiquitination of NFATc3. Consistently, the half-life of the NFATc3-EallA mutant, measured after inhibition of protein synthesis with cycloheximide, was significantly increased compared to WT NFATc3 (Fig. 6C,D). Taken as a whole, these data suggest that SUMOylation of NFATc3 favours its ubiquitination and subsequent degradation.

Trim39 acts as a SUMO-targeted E3 ubiquitin-ligase for NFATc3

To better understand the mechanisms underlying the regulation of NFATc3 by SUMO, we examined whether mutation of its three consensus SUMOylation sites could affect its ubiquitination by Trim39. Indeed, the ubiquitination level of the NFATc3-EallA mutant was decreased compared to WT NFATc3 when co-expressed with Trim39 in Neuro2A cells (Fig. 7A). To determine whether this could be due to a reduced interaction between NFATc3 and Trim39, co-immunoprecipitation experiments were performed. The amount of Trim39 coprecipitated with NFATc3-EallA was decreased compared to the amount of Trim39 coprecipitated with WT NFATc3 (Fig. 7B left panel). Consistently, the amount of NFATc3 coprecipitated with Trim39 was decreased when its three SUMOylation sites were mutated (Fig. 7B right panel). Therefore, these data suggest that Trim39 binds and ubiquitinates preferentially SUMOylated forms of NFATc3.

Proteins interacting non-covalently with SUMO generally harbor SUMO-interacting motifs (SIMs). These motifs typically consist of three hydrophobic residues in a sequence of four amino acids, sometimes flanked by acidic or phosphorylated residues (Kerscher, 2007). Using the web-based tool GPS-SUMO (Q. Zhao et al., 2014), we identified three putative SIMs in the Trim39 sequence, which are conserved from mouse to human. We named these motifs SIM1 (39-PVII-42, located in the RING domain), SIM2 (125-VCLI-128, in the B-Box domain) and SIM3 (211-LLSRL-215, in the coiled-coil domain). These three putative SIMs exhibit the highest predictive scores with GPS-SUMO. Two of them, SIM1 and SIM2, are also predicted with a high score by the JASSA bioinformatics tool (Beauclair et al., 2015). Trim39 constructs were generated in which most residues of the three SIMs were mutated to Ala (respectively into mSIM1: 39-PAAA-42, mSIM2: 125-AAAA-128 and mSIM3: 211-AAARA-215). To confirm the ability of Trim39 to bind SUMO and to determine the impact of these mutations, we
conducted GST pull-down experiments using purified recombinant proteins. Interestingly, GST-Trim39 could bind di-, tri-, tetra- and higher-order SUMO-2 chains but not free SUMO-2, whereas GST alone showed no interaction (Fig. 7C). Single mutations of SIM1 and SIM2 had no significant effect, either individually (Fig. 7C) or together (Fig. S3). In contrast, mutation of SIM3 strongly reduced the SUMO-binding ability of Trim39 (Fig. 7C), an effect which was not significantly modified by combination with single SIM1 mutation, and only slightly increased by combination with single SIM2 and double SIM1/SIM2 mutations (Fig. S3), as previously described for the SUMO-target E3 ubiquitin-ligase Arkadia/RNF11 (Erker et al., 2013). These data suggest that SIM3 plays a pivotal role in the binding of Trim39 to SUMO chains. Consistently, mutation of SIM3 reduced the ability of Trim39 to interact with NFATc3 in co-immunoprecipitation experiments (Fig. 7D). As for SUMO-2 chain binding (Fig. 7C), the concomitant mutation of SIM1, SIM2 or both, together with SIM3, did not significantly modify the binding of Trim39 to NFATc3 (Fig. 7D). Moreover, SIM3 mutation reduced the ability of Trim39 to ubiquitinate NFATc3 in Neuro2A cells (Fig. 7E).

Collectively, these data strongly suggest that Trim39 acts as a SUMO-targeted E3 ubiquitin-ligase for NFATc3 by preferentially binding the SUMOylated forms of NFATc3 through its SIM, in order to mediate their ubiquitination.

**SUMOylation and Trim39 modulate the pro-apoptotic effect of NFATc3 in neurons**

In a previous study, we have shown that overexpression of NFATc3 in primary cultures of cerebellar granule neurons (CGNs) aggravates apoptosis induced by KCl deprivation (Mojsa et al., 2015). Primary CGNs represent one of the best characterized *in vitro* models of neuronal apoptosis (Contestabile, 2002). These neurons survive in the presence of serum and depolarizing concentrations of KCl (25 mM) that mimic the neuronal activity required for their survival *in vivo* (Ikonomidou et al., 1999). They undergo apoptosis following withdrawal of serum and lowering of KCl to 5 mM (K5) (D’Mello et al., 1993), which recapitulates the programmed cell death naturally occurring in the cerebellum during post-natal development (Wood et al., 1993). We used this model to examine whether mutation of the SUMOylation sites of NFATc3, which increases its stability (Fig. 6D) by reducing its interaction with Trim39 (Fig. 7B) and its ubiquitination (Fig. 7A), could have an impact on its pro-apoptotic effect in CGNs. As shown previously (Mojsa et al., 2015), we confirmed that KCl deprivation-induced apoptosis is significantly increased in CGNs transfected with WT GFP-NFATc3 compared to GFP, as shown by the increased number of apoptotic/condensed nuclei (Fig. 8A,B). Interestingly,
neuronal apoptosis was further increased in neurons overexpressing GFP-NFATc3-EallA compared to WT GFP-NFATc3 (Fig. 8A,B). Consistently, efficient silencing of Trim39 using a lentivirus expressing a specific shRNA (Fig. 8C) significantly aggravated apoptosis compared to neurons transduced with an unrelated control shRNA (Fig. 8D,E). Our data therefore strongly suggest that SUMO and Trim39 negatively regulate the pro-apoptotic function of NFATc3, most likely by reducing its stability and thereby its activity as a transcription factor.

**Discussion**

In contrast to calcium/calcineurin-mediated NFAT nuclear translocation, the regulation of NFAT protein stability by the ubiquitin-proteasome system has been poorly studied. Independent studies have suggested that certain E3 ubiquitin-ligases may be responsible for ubiquitination and proteasomal degradation of different NFAT members: HDM2 for NFATc2 in breast and pancreatic cancers (Singh et al., 2011; Yoeli-Lerner et al., 2005); Cbl-b, c-Cbl, VHL or KBTBD11/Cullin3 for NFATc1 during osteoclastogenesis (J. H. Kim et al., 2010; X. Li et al., 2015; Narahara et al., 2019; Youn et al., 2012); CHIP for NFATc3 in LPS-induced cardiomyopathies (Chao et al., 2019). However, no formal demonstration has been made to establish that these proteins are genuine NFAT E3 ubiquitin-ligases, with the exception of HDM2 for NFATc2 (Yoeli-Lerner et al., 2005). In the present study, we provide the first formal identification of an NFATc3 E3 ubiquitin-ligase. Indeed, we show several lines of evidence demonstrating that Trim39 is indeed an E3 ubiquitin-ligase for NFATc3. First, we found a physical interaction between endogenous or overexpressed Trim39 and NFATc3 proteins. Second, Trim39 ubiquitinated NFATc3 *in vitro*. Third, overexpression of WT Trim39, but not of its inactive RING mutant, increased the ubiquitination level of NFATc3 in cells. In contrast, silencing of Trim39 decreased NFATc3 ubiquitination. Finally, Trim39 overexpression decreased the protein level of NFATc3 whereas the silencing of endogenous Trim39 increased it, suggesting that Trim39-mediated ubiquitination of NFATc3 targets it for proteasomal degradation. As a physiological consequence, overexpressed Trim39 resulted in reduced transcriptional activity of NFATc3 without affecting its nuclear translocation. Conversely, silencing of endogenous Trim39 increased both the expression of a NFATc3 target gene and its pro-apoptotic effect in neurons. Taken together, these data strongly suggest that Trim39 modulates neuronal apoptosis by acting as a physiological E3 ubiquitin-ligase for NFATc3. This does not exclude the possibility that other NFATc3 E3 ubiquitin-ligases exist, notably in other cell types, such as CHIP in cardiomyocytes (Chao et al., 2019). Nevertheless, NFATc3
ubiquitination is deeply decreased following Trim39 knock-down in Neuro2A cells, suggesting that Trim39 is the major E3 ubiquitin-ligase for NFATc3 in these cells. Our present data show that Trim17 inhibits the ubiquitination of NFATc3 mediated by Trim39. Indeed, the increase in the ubiquitination level of NFATc3 due to overexpression of Trim39 was abolished by the co-transfection of Trim17. Both Trim39 and Trim17 belong to the family of TRIM proteins which forms one of the largest classes of RING-containing E3 ubiquitin-ligases (Meroni & Diez-Roux, 2005), comprising 82 members in humans (Qiu et al., 2020). TRIM proteins are characterized by their N-terminal tripartite motif that consists of a RING domain, one or two B-box domains and a coiled-coil domain, invariably ordered in this sequence from N- to C-termini (Esposito et al., 2017; Reymond et al., 2001). In addition to this common motif, TRIM proteins generally exhibit a C-terminal domain that varies from one member to another and categorizes them into different subtypes (Short & Cox, 2006). This C-terminal domain, which is a PRY-SPRY domain for both Trim17 and Trim39, generally mediates target recognition and specificity (Esposito et al., 2017; Y. Li et al., 2014). While the RING domain confers an E3 ubiquitin-ligase activity by binding ubiquitin-loaded E2 ubiquitin-conjugating enzymes, the B-box and especially the coiled-coil domain are involved in the formation of homo- or hetero-dimers or multimers (Koliopoulos et al., 2016; Y. Li et al., 2014; Napolitano & Meroni, 2012; Sanchez et al., 2014). As homo-oligomerization seems to be necessary for the E3 ubiquitin-ligase activity of TRIM proteins (Koliopoulos et al., 2016; Streich et al., 2013; Yudina et al., 2015), Trim17 may inhibit Trim39-mediated ubiquitination of NFATc3 by forming inactive hetero-oligomers with Trim39. Indeed, we show here that Trim17 and Trim39 physically interact with each other. In a similar way, we have previously shown that TRIM17 inhibits the activity of two other TRIM proteins to which it is able to bind: TRIM41 (Iréna Lassot et al., 2018) and TRIM28 (Lionnard et al., 2019). It is interesting to note that TRIM39 and TRIM41 are very close from a phylogenetic point of view (Qiu et al., 2020; Sardiello et al., 2008), suggesting that common mechanisms could be involved in their inhibition by Trim17. The inhibitory effect of Trim17 might result from two mechanisms that are not mutually exclusive. First, the formation of hetero-dimers or hetero-multimers with Trim17 may inhibit the intrinsic E3 ubiquitin-ligase activity of its TRIM partner, possibly by preventing the binding of the E2 enzyme. Indeed, we show in the present study that TRIM17 prevents the auto-ubiquitination of TRIM39 in vitro, similarly to what we have previously shown for the inhibition of TRIM41 by TRIM17 (Iréna Lassot et al., 2018). Second, Trim17 may prevent the
binding of the substrate to its TRIM partner. Indeed, we show here that Trim17 reduces the
interaction between Trim39 and NFATc3, as determined by both co-IP of overexpressed
proteins and proximity ligation of endogenous proteins (PLA). These results are similar to the
effect of TRIM17 on the interaction between TRIM41 or TRIM28 and their respective
substrates (Iréna Lassot et al., 2018; Lionnard et al., 2019). In the three cases, Trim17 is able to
bind both the substrate and the TRIM partner, suggesting that Trim17 could reduce their
interaction either by directly binding the substrate in a competitive manner, or because the
formation of a hetero-dimer hinders the accessibility of the TRIM partner to the substrate.
Further experiments are required to determine the structural determinants of the inhibitory
effect of Trim17 on other TRIM proteins. Nevertheless, it is unlikely that Trim17 inhibits
Trim39-mediated ubiquitination of NFATc3 by associating with a deubiquitinating enzyme
(DUB), as shown for other TRIM proteins (Hao et al., 2015; Nicklas et al., 2019). Indeed,
TRIM17 is able to inhibit the in vitro ubiquitination of NFATc3 mediated by TRIM39, in a
completely acellular medium in the absence of any DUB. It is also clear that Trim17 does not
inhibit Trim39 by inducing its ubiquitination and subsequent degradation. Indeed, Trim17
rather decreases the ubiquitination level of Trim39 both in vitro and in cells. Moreover, it is
interesting to note that, TRIM39 reciprocally decreases the in vitro auto-ubiquitination of
TRIM17, further suggesting that Trim17 and Trim39 form inactive hetero-dimers or hetero-
multimers, in which the E3 ubiquitin-ligase activity of the two partners is inhibited. Formation
of inactive hetero-oligomers might be favored by the fact that Trim17 and Trim39 belong to
the same phylogenetic group (Qiu et al., 2020; Sardiello et al., 2008), as hetero-interactions are
more often observed among closely related TRIM family members (Napolitano & Meroni,
2012).

SUMOylation of proteins changes their non-covalent interactions in various ways, leading to
alterations in their subcellular localization and function. As such, SUMO modification plays
crucial roles in a wide range of cellular processes (Henley et al., 2018; Yau et al., 2020; X.
Zhao, 2018). SUMOylation has also emerged as an important regulator of protein stability
(Liebelt & Vertegaal, 2016). Consistently, our data clearly indicate that SUMOylation of
NFATc3 promotes its ubiquitination and subsequent degradation. Indeed, mutation of the three
SUMOylation consensus sites of NFATc3 decreased its ubiquitination level, increased its half-
life and aggravated its pro-apoptotic effect in neurons. As the SUMOylation sites were modified
in order to specifically prevent SUMOylation without affecting a putative ubiquitination of the
acceptor Lys residues, our results unambiguously designate SUMOylation as the post-
translational modification implicated in these effects. This mechanism could be conserved in
other members of the NFAT family. Indeed, effective SUMOylation of NFAT proteins has been
described, notably for NFATc1 and NFATc2 (Nayak et al., 2009; Terui et al., 2004). Although
the functional consequences of NFAT SUMOylation that have been reported so far are rather
related to their nuclear translocation and transactivation activity (E. T. Kim et al., 2019; Nayak
et al., 2009; Terui et al., 2004; Vihma & Timmusk, 2017), it might also influence the stability
of these proteins. Indeed, Singh et al. reported that the double mutation of Lys684 and Lys897
in murine NFATc2 prevents its ubiquitination and degradation induced by zoledronic acid
(Singh et al., 2011). One possible conclusion is that NFATc2 is normally ubiquitinated on these
Lys residues (Singh et al., 2011). However, as these two Lys residues have been shown to be
SUMOylated (Terui et al., 2004), an alternative plausible explanation is that SUMOylation of
NFATc2 on these consensus sites might be necessary for the recognition by its E3 ubiquitin-
ligase. In line with this hypothesis, the protein level of different human NFATc1 and NFATc2
isoforms have been reported to be increased by the double K/R mutation (lysine to arginine
substitution) of their C-terminal SUMOylation sites (Vihma & Timmusk, 2017). However,
further investigation is required to demonstrate that SUMO indeed plays a role in these effects.

A few SUMO-targeted E3 ubiquitin-ligases (STUbLs) have been described (Geoffroy & Hay,
2009; Prudden et al., 2007; Sriramachandran & Dohmen, 2014). These proteins generally
combine two features: a RING domain, that confers them an E3 ubiquitin-ligase activity, and
SUMO interacting motifs (SIMs) that mediate their preference for SUMOylated substrates
(Geoffroy & Hay, 2009; Prudden et al., 2007; Sriramachandran & Dohmen, 2014). Inhibition
of the proteasome leads to an important accumulation of high molecular weight SUMO-
modified proteins in yeast and human cells (Bailey & O’Hare, 2005; Uzunova et al., 2007),
suggesting that ubiquitination and degradation of SUMOylated proteins mediated by STUbLs
play an important role in proteostasis. However, only two STUbLs have been identified so far
in mammals: RNF4 and Arkadia/RNF111 (Branigan et al., 2019; Sriramachandran & Dohmen,
2014), which may not be sufficient to account for the regulation of all SUMOylated proteins.
Therefore, additional STUbLs probably remain to be discovered. Here we provide a series of
arguments indicating that Trim39 acts as a STUbL for NFATc3. First, Trim39 is able to bind
SUMO chains but not free SUMO in vitro. Second, we identified a SIM in the sequence of
Trim39. Indeed, mutation of this motif strongly decreases its capacity to bind SUMO-2 chains
in vitro. Third, the binding and the ubiquitination of NFATc3 mediated by Trim39 is reduced
by mutation of this SIM in Trim39. Finally, Trim39 binds and ubiquitinates preferentially the SUMOylated forms of NFATc3.

Most of the STUbLs studied so far bear multiple SIMs, which mediate cooperative binding to multiple SUMO units, thereby providing a preference for substrates with SUMO chains (Sriramachandran & Dohmen, 2014). Among the three SIMs predicted in the Trim39 sequence with a high score, only one is really involved in the binding of Trim39 to purified SUMO chains and SUMOylated NFATc3 in cells. SIMs are characterized by a loose consensus sequence and some non-canonical SIMs have been described (Kerscher, 2007; Sriramachandran & Dohmen, 2014). Therefore, it is possible that another SIM in Trim39, that was not identified or was not credited with a high predictive score by GPS-SUMO or JASSA, may participate in the binding of Trim39 to SUMO. It is also possible that one SIM is sufficient to fulfill this function, as has been reported for other STUbLs (Erker et al., 2013; Parker & Ulrich, 2012). For example, among the three SIMs identified in Arkadia/RNF111, only one has been shown to be crucial for its interaction with SUMO-2 chains, exactly like SIM3 in Trim39 (Erker et al., 2013).

Moreover, the activity of TRIM proteins generally involves homo-dimerization or homo-multimerization (Koliopoulos et al., 2016; Streich et al., 2013; Yudina et al., 2015). It is therefore possible that in its multimerized form, Trim39 harbors several SIMs in close proximity. Indeed, SIM3 is located in the coiled-coil domain of Trim39 that is expected to form antiparallel dimers or higher-order multimers, as reported in other TRIM proteins (Koliopoulos et al., 2016; Y. Li et al., 2014; Napolitano & Meroni, 2012; Sanchez et al., 2014). Therefore, the unique active SIM of one molecule of Trim39 may cooperate with the SIM of other Trim39 molecules, in multimers, for binding SUMO chains. Alternatively, another binding site, such as the RING domain, may cooperate with the SUMO-SIM interaction to bind the substrate, as has been reported for the Drosophila STUbL Degringolade (Abed et al., 2011). Trim39 may also bind NFATc3 by a dual mechanism, involving both SUMO-dependent and SUMO-independent recognition, as shown for viral STUbLs (Boutell et al., 2011; Wang et al., 2011). Indeed, we found that the NFATc3/Trim39 interaction is decreased but not completely abrogated, in co-immunoprecipitation experiments, by mutation of either the SUMOylation sites in NFATc3 or SIM3 in Trim39. Further studies are required to fully characterize the mechanisms mediating the SUMO-dependent interaction of NFATc3 with Trim39.

TRIM proteins play significant roles in a wide variety of cellular processes including cell cycle, differentiation, apoptosis, autophagy, transcription, DNA repair, innate immunity and anti-viral defense (Hatakeyama, 2017; Venuto & Merla, 2019). As a consequence, mutations of TRIM
genes have been implicated in several human diseases such as cancer, auto-immune diseases, rare genetic disorders, infectious diseases and neurodegenerative diseases (Hatakeyama, 2017; Meroni, 2020; Watanabe & Hatakeyama, 2017). In particular, Trim39 has been shown to regulate cell cycle progression by directly mediating the ubiquitination of p53 (Zhang, Huang, et al., 2012) and by indirectly modulating the protein level of p21 (Zhang, Mei, et al., 2012). Trim39 has also been implicated in the negative regulation of NFκB signaling (Suzuki et al., 2016). In the present study, we show that silencing of Trim39 enhances apoptosis triggered by survival-factor deprivation in primary cultures of neurons. As Trim39 induces NFATc3 degradation and as NFATc3 aggravates neuronal apoptosis, these data suggest that silencing of Trim39 favors apoptosis of neurons by stabilizing NFATc3. However, it does not exclude additional effects of Trim39 on apoptosis regulation. For example, silencing of Trim39 has been reported to increase nutlin3a-induced apoptosis in several p53-positive cancer cell lines, presumably by stabilizing the pro-apoptotic factor p53 (Zhang, Huang, et al., 2012). Trim39 knock-down has also been reported to aggravate apoptosis following a genotoxic stress in HCT116 cells (Zhang, Mei, et al., 2012). Conversely, in p53 null cell lines, silencing of Trim39 reduces DNA damage-induced apoptosis (S. S. Lee et al., 2009; Zhang, Huang, et al., 2012). The latter effect is likely due to the ability of Trim39 to directly inhibit APC/C_Cdh1-mediated degradation of the pro-apoptotic protein MOAP-1 (Huang et al., 2012; S. S. Lee et al., 2009). These data, taken together with the present study, place Trim39 as a key factor in several processes regulating apoptosis, the final outcome depending on its targets or binding partners present in the cell and the nature of the cellular stress. Similarly, we and others have reported that Trim17 plays an important role in apoptosis regulation (I. Lassot et al., 2010; Lionnard et al., 2019; Magiera et al., 2013; Mojsa et al., 2015; Song et al., 2017). Notably, we have shown that Trim17 is both sufficient and necessary to trigger apoptosis in primary neurons (I. Lassot et al., 2010), at least in part by mediating the ubiquitination and subsequent degradation of the anti-apoptotic protein Mcl-1 (Magiera et al., 2013). Trim17 may also modulate neuronal apoptosis by acting on NFATc3 through antagonistic mechanisms. On one hand, we have previously shown that Trim17 can prevent the nuclear translocation and transcriptional activity of NFATc3 (Mojsa et al., 2015) and should therefore inhibit its pro-apoptotic effect. On the other hand, we show here that Trim17 can inhibit Trim39-mediated ubiquitination and degradation of NFATc3 and should therefore aggravate its pro-apoptotic effect by increasing its protein level. Moreover, we have identified Trim17 as a target gene of NFATc3 (Mojsa et al., 2015). The effects of Trim17 on the protein level and activity of NFATc3 should therefore influence its own expression, creating both a negative and a positive feedback loop and
eventually resulting in fine tuning of neuronal apoptosis. Further investigation will determine whether these mechanisms can be manipulated for therapeutic purposes to prevent neuronal loss in neurodegenerative diseases.

Materials and Methods

Materials

Culture media were from Thermo Fisher Scientific. Fetal calf serum, other culture reagents, protease inhibitor cocktail, DAPI, PMA, A23187, cycloheximide, N-ethylmaleimide (NEM), MG-132, puromycin, anti-Flag M2 affinity gel beads (#A2220) and other chemicals were from Sigma-Aldrich. Protein G-agarose and protein A-agarose beads were from Roche. GFP-Trap®-A beads were from Chromotek (Planneg-Martinsried, Germany). Rat monoclonal anti-HA antibody (clone 3F10; #11867432001), mouse monoclonal anti-Flag antibody (clone M2, #F3165), mouse monoclonal anti-tubulin antibody (clone DM1A, #T6199), rabbit anti-TRIM17 antibody (#AV34547) and rabbit IgG (#I5006) were from Sigma-Aldrich. Rabbit anti-GFP antibody (#TP401) was from Torrey Pines Biolabs Inc. (Houston, TX USA). Mouse monoclonal antibody against actin (clone C4) was from Millipore (#MAB1501). Mouse polyclonal antibody against NFATc3 was from Proteintech (18222-1-AP). Mouse monoclonal antibody against Trim39 was from Origene (#TA505761). Rabbit polyclonal antibody against Trim39 was from Proteintech (#12757-1-AP). Monoclonal mouse antibody against SUMO-2 (clone #8A2) was purified from hybridomas obtained from the Developmental Studies Hybridoma Bank. Fluorescent and horseradish peroxidase-conjugated goat anti-rabbit, anti-rat and anti-mouse secondary antibodies were from Thermo Fisher Scientific and Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA), respectively.

Cell culture and transient transfection

Lenti-X 293 T (Clontech), Neuro2A (mouse neuroblastoma) and Baby Hamster Kidney (BHK) cell lines were grown in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose supplemented with 10% fetal bovine serum and penicillin-streptomycin 100 IU/ml-100 µg/ml. Cells were transfected with plasmids using GenJet™ in vitro transfection reagent (Ver. II) pre-optimized and conditioned for transfecting Neuro2A and BHK-21 cells respectively (SignaGen
laboratories, Ijamsville, MD) according to the manufacturer’s instructions. Neuro2A cells were transfected with siRNAs using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) following the manufacturer’s instruction. For one 35 mm dish, 2.5 µl of transfection reagent was used with 25 pmoles of siRNA. The sequences of the siRNAs used were as follows: siTrim39#1 5' CCAAGCGGGTAGCATATT 3'; siTrim39#2 5' GCGTCAAGTTTGTGGAGACAA3'; siRNA ctrl (targeting Luciferase gene): 5' CGTACGCGGAATACTTCGA 3'.

Primary cultures of CGNs were prepared from 7-day-old murine pups (C57Bl/6 J mice) as described previously (I. Lassot et al., 2010). Briefly, freshly dissected cerebella were dissociated by trypsinization and mechanical disruption, and plated in Basal Medium Eagle (BME) medium supplemented with 10% fetal bovine serum, 2 mM L-Gln, 10 mM HEPES, penicillin-streptomycin 100 IU/ml-100 µg/ml and 20 mM KCl. Primary CGNs, grown on glass coverslips in 24-well plates, were transfected at DIV 5 with 2 µg of plasmids using a calcium phosphate protocol optimized for neuronal cultures as previously described (I. Lassot et al., 2010).

Silencing of Trim39 using shRNA-expressing lentiviruses

The HIV-derived lentiviral vectors pLKO.1 containing control shRNAs respectively against eGFP and Luciferase (SHC005, SHC007) and the shRNAs TRCN0000037281 (shRNA Trim39#1), TRCN0000037282 (shRNA Trim39#2) and TRCN0000438509 (shRNA Trim39#3) were from Sigma-Aldrich. Lentiviral particles were produced as previously described (Iréna Lassot et al., 2018). Neuro2A cells were transduced one day after plating. The lentiviral preparations were added directly to the culture medium for 8 h (approximately 500 ng p24 per million neurons, approximately 100 ng p24 per million Neuro2A cells). Cells were then replaced in fresh medium. Culture was continued until 6 days in vitro for CGNs. Neuro2A cells were maintained in culture for 24 h after transduction and then selected using 2 µg/ml puromycin for an additional 48 h.

Expression vectors and site directed mutagenesis

The following plasmids were described previously: pCI-GFP, pCS2-3×HA-NFATc3, pCS2-3×HA-NFATc3-KalIR, pCS2-GFP-NFATc3 and pCI-Trim17-GFP (Mojsa et al., 2015). All the
primers used to generate the constructs described below are listed in supplementary Table1. The sequences of all the constructs were confirmed by automatic sequencing. Single point mutations in the SUMOylation-consensus sites of NFATc3 (E437A, E706A and E1015A) or their double and triple combinations (E437A/E706A, E706A/E1015A and E437A/E706A/E1015A=EallA) were obtained by site-directed mutagenesis of pCS2-3×HA-NFATc3 using the QuickChange® II XL kit (Agilent Technologies) using the indicated primers. To increase the expression of NFATc3, HA-NFATc3 and HA-NFATc3-EallA from respective pCS2-3×HA expression vectors were first sub-cloned between XhoI and Nhel sites of the pCDNA3.1 plasmid, and then sub-cloned between SalI and Nhel sites of the pCI plasmid, to generate pCI-3×HA-NFATc3 and pCI-3×HA-NFATc3-EallA. The plasmid pCS2-GFP-NFATc3-EallA was obtained by removing the WT NFATc3 cDNA from the plasmid pCS2-GFP-NFATc3 and by replacing it with NFATc3-EallA between EcoRI and XhoI sites of the plasmid. The cDNA of mouse Trim39 (GenBank: NM_024468) was amplified, from primary CGN cDNAs, by using PCR with the indicated primers. Amplicons were then cloned into pCI-3×Flag plasmid between EcoRI and XbaI sites to obtain mouse pCI-3×Flag-Trim39. pGEX-4T1-Trim39-ΔRING mutant was generated by PCR amplification of Trim39 coding region using pCI-3×Flag-Trim39 as template and the indicated primers. Then, the amplicons were cloned between EcoRI and XhoI sites of the plasmid pGEX4T1. To obtain pCI-3×Flag-Trim39-ΔRING, the insert was released from the plasmid pGEX4T1-Trim39-ΔRING and sub-cloned between EcoRI and NotI sites of the plasmid pCI-3×Flag. The following Trim39 SIM mutants were obtained by site-directed mutagenesis using pCI-3×Flag-Trim39 as a template and the indicated primers: single mutants mSIM1 (PVII→PAAA), mSIM2 (VCLI→ACAA) and mSIM3 (LLSRL→AAARA); double mutants mSIM12, mSIM13 and mSIM23; and triple mutant mSIM123. The expression vector pmCherry-C1 was purchased from Takara Bio Inc. (#632524). The plasmid mouse pCI-Trim39-mCherry was obtained by recombinant PCR. The first PCR was performed with the indicated primers using pCI-3×Flag-Trim39 as a template. The second PCR was performed with the indicated primers using pmCherry-C1 as a template. The amplicons from both PCRs were purified, mixed and used as template for the recombinant PCR (third PCR) with the indicated primers. The resulting amplicon was cloned between EcoRI and XbaI sites of the empty pCI plasmid to obtain pCI-Trim39-mCherry.

In order to produce recombinant N-terminal GST-tagged Trim39 protein in *Escherichia coli*, the pGEX-4T1-Trim39 construct was produced by PCR amplification of the Trim39 coding region using pCI-3×Flag-Trim39 as a template and the indicated primers. The PCR products
were cloned between the EcoRI and XhoI sites of the pGEX-4T1 expression vector (GE-Healthcare). The mutant GST-Trim39-C49S/C52S was generated by site-directed mutagenesis using GST-Trim39 as a template and the indicated primers. SIM1, SIM2 and SIM3 GST-Trim39 mutants were generated by site-directed mutagenesis using GST-Trim39 as a template and the same primers as for pCI-3×Flag-Trim39 SIM mutants. The cDNA of human TRIM39 (GenBank: NM_172016.2), N-Terminally fused to a histidine tag in the plasmid pET-15, and the cDNA of human TRIM17 (GenBank: NM_016102), C-terminally fused to GFP in the pEGFP plasmid were obtained from the ORFeome library of the Montpellier Genomic Collections facility. Human TRIM17 was first amplified by PCR using p-TRIM17-EGFP as a template and indicated primers, and the amplicons were cloned between the EcoRI and SalI sites of the pGEX-4T1 expression vector. In order to produce recombinant N-terminally MBP-tagged TRIM17 protein in *Escherichia coli*, the pMAL-c2-TRIM17 plasmid was generated by subcloning. The insert was released from the plasmid pGEX-4T1-TRIM17 and sub-cloned between the EcoRI and SalI sites of the plasmid pMAL-c2 to obtain pMAL-c2-TRIM17.

**Protein extraction and western blot analysis**

Cells were harvested in lysis buffer A (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 5 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 5 mM EDTA, 10 mM NEM, 20 µM MG-132, and protease inhibitor cocktail) supplemented with 1% NP-40 and homogenized by thorough vortexing. Cell debris were removed by centrifugation at 1000 × g for 5 min at 4°C and the protein concentration of the resulting supernatant was estimated using the BCA protein assay kit (Thermo Fisher Scientific) with bovine serum albumin as the standard. Total lysates were diluted in 3 × Laemmli sample buffer and incubated at 95°C for 5 min. Proteins were separated by 8% to 12% SDS–PAGE and transferred to Immobilon-P PVDF membrane (Millipore). Blocking and probing with antibodies were performed as previously described (Iréna Lassot et al., 2018). Visualization of immunoreactive proteins was performed using horseradish peroxidase-linked secondary antibodies and Covalight enhanced chemiluminescent substrate (Covalab, Bron, France) or Immobilon® Western (Millipore). Membranes were revealed using films or Amersham Imager 680 (GE Healthcare). When necessary, membranes were stripped using Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific) and re-probed with additional antibodies.
Co-immunoprecipitation

Following transfection with the indicated plasmids for 24 h, Neuro2A or BHK cells were incubated for 5 h with 10-20 µM MG-132. They were then homogenized in lysis buffer A, supplemented with 1% NP-40 for immunoprecipitation with anti-HA, 0.5% NP-40 for immunoprecipitation with GFP-Trap-A and 1% Triton X-100 for immunoprecipitation with anti-Flag. For anti-HA and anti-Flag immunoprecipitation, cell lysates (500 µl) were centrifuged at 300 × g for 5 min at 4°C. The resulting supernatants were pre-cleared by rotation for 1-3 h at 4°C with 15 µl protein G-agarose beads and then rotated overnight at 4°C with 25 µl protein G agarose beads together with 7 µl anti-HA antibody or with 30 µl anti-Flag M2 affinity gel beads. The beads were recovered by centrifugation and washed four times with 1 ml of lysis buffer A without detergent and containing 0.3 M NaCl for anti-HA or 0.5 M NaCl for anti-Flag (instead of 150 mM NaCl). For GFP-Trap precipitation, cell lysates (200 µl) were diluted with 300 µl dilution buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 5 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 0.5 mM EDTA, 20 µM MG-132, 10 mM NEM and protease inhibitor cocktail) and cell debris were removed by centrifugation. Resulting supernatants were rotated for 2 h at 4°C with 10-25 µl GFP-Trap®-A beads to immunoprecipitate proteins fused to GFP. Beads were recovered by centrifugation and washed four times with dilution buffer. Material bound to the protein G agarose, anti-Flag M2 affinity gel beads or GFP-Trap beads was eluted by the addition of 3 × Laemmli sample buffer and incubation at 95°C for 5 min. Precipitated proteins were analyzed by western blot.

In situ proximity ligation assay

Neuro2A cells seeded onto glass coverslips, were left untreated or transfected with pCI-Flag-Trim39, pCI-GFP or pCI-Trim17-GFP for 24 h, and then treated with 10 µM MG-132 for 4 h. Cell were fixed with 4% paraformaldehyde for 20 min at room temperature, washed with 0.1 M Gly (pH = 7.11), permeabilized with 0.2% Triton X-100 in PBS for 10 min and washed with PBS. The interaction between endogenous Trim39 and endogenous NFATc3, in the presence or absence of GFP or Trim17-GFP, was detected using the Duolink® In Situ kit (Olink® Bioscience, Uppsala, Sweden), according to the manufacturer’s instructions, as described previously (Mojsa et al., 2015). Briefly, cells were successively incubated with blocking solution for 60 min at 37°C, with primary antibodies against Trim39 (Origene, 1:400) and NFATc3 (Proteintech, 1:200) overnight at 4°C and with secondary antibodies conjugated with
oligonucleotides (PLA probe MINUS and PLA probe PLUS) for 1 h at 37°C. The cells were then incubated with two connector oligonucleotides together with DNA ligase for 30 min at 37°C. If the two secondary antibodies are in close proximity, this step allows the connector oligonucleotides to hybridize to the PLA probes and form a circular DNA strand after ligation. Incubation, for 100 min at 37°C, with DNA polymerase consequently leads to rolling circle amplification (RCA), the products of which are detected using fluorescently-labeled complementary oligonucleotides. Cells were washed with Duolink In Situ wash buffers following each of these steps. In the last wash, 1 µg/ml DAPI was added to the cells for 5 min at room temperature in the dark to stain the nuclei and coverslips were set in Mowiol (polyvinyl alcohol 4-88, Fluka), on glass slides. Cells were analyzed using a confocal Leica SP5-SMD microscope, with a LEICA 63x/1.4 OIL HCX PL APO CS objectives. Images were acquired by the Confocal head TCS SP5 II using the Leica Application Suite X software. Images were processed using Image J. When indicated, z-stacks of images were submitted to maximum intensity projection. The number of dots per transfected cell was estimated in one slice, in around 100 cells in each condition, with an automated procedure using plugins from the Image J software.

Immunofluorescence

BHK and Neuro2A cells were seeded onto glass coverslips. Primary CGNs were cultured on coverslips previously coated with laminin (16.67 µg/ml) and poly-D-lysine (33.3 µg/ml). Cells and neurons were treated as described in the figure legends and then fixed with 4% paraformaldehyde. Overexpressed HA-NFATc3 and endogenous Trim39 were detected using anti-HA (1:500) and anti-Trim39 (from Proteintech 1:200, from Origene 1:400) antibodies respectively, as described previously (Iréna Lassot et al., 2018). GFP and mCherry-fused proteins were visualized by fluorescence and nuclei were stained with DAPI. Coverslips were analyzed by conventional or confocal microscopy, as mentioned in the figure legends. Image acquisition and analysis were performed on work stations of the Montpellier imaging facility (Leica DM600 for conventional microscopy, Leica SP5-SMD for confocal microscopy). For quantification of NFATc3 nuclear localization, BHK cells with predominant cytoplasmic or nuclear localization of NFATc3 were counted, in a blinded manner, among double GFP/HANFATc3 positive cells. At least 100 double positive cells were scored for each experiment and each condition.
In vivo ubiquitination of NFATc3

Neuro2A or BHK cells cultured in 60 mm or 100 mm dishes were transfected with pCI-HA-NFATc3 together with a plasmid expressing eight His<sub>6</sub>-tagged ubiquitin (His-Ub), or empty pCI, in the presence or the absence of pCI-Flag-Trim39, pCI-Flag-Trim39-ΔRING, pCI-Trim17-GFP or a combination of these plasmids. Following 24 h transfection, the medium was supplemented with 20 µM MG-132 for 6 h. Then, cells were harvested in 1 ml PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> supplemented with 10 µM MG-132. In some experiments, a 100 µl sample of the homogenous cell suspension was taken for input analysis and transfection efficiency control. After centrifugation, the pellet from the remaining 900 µl cell suspension was homogenized in 1 ml lysis buffer B (6 M guanidinium-HCl, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl [pH 8.0]) supplemented with 5 mM imidazole, 510 mM β-mercaptoethanol, 0.5 M NaCl and 10 mM NEM. The lysate was sonicated, cleared by centrifugation at 1,500 × g for 5 min at room temperature. In some experiments, input analysis was made at this stage by precipitating 50 µl of the resulting supernatants with TCA. The remainder of each extract was added to 40 µl magnetic nickel beads (MagneHis™ Ni-Particles, Promega). Beads were rotated for 2 h at room temperature to purify ubiquitinated proteins and washed once with lysis buffer B supplemented with 20 mM imidazole, 0.5 M NaCl and 10 mM NEM, once with 8 M urea, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl (pH 8.0), 20 mM imidazole, 0.5 M NaCl and 10 mM NEM, three times with 8 M urea, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl (pH 6.3), 20 mM imidazole, 0.5 M NaCl, 10 mM NEM, 0.2% Triton X-100, once with 8 M urea, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl (pH 6.3), 20 mM imidazole, 0.5 M NaCl, 10 mM NEM, 0.1% Triton X-100 and once with 8 M urea, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl (pH 6.3), 10 mM imidazole, 10 mM NEM. Materials bound to the beads were eluted by the addition of 3 × Laemmli sample buffer and boiling for 5 min. These purification products and initial total lysates were resolved by SDS-PAGE and analyzed by immunoblotting.

Production of recombinant TRIM proteins

BL21-CodonPlus®(DE3)-RP competent cells (Agilent) were transformed with the expression vectors pGEX-4T1 expressing GST-Trim39 fusion proteins (WT and mutants). Protein expression was induced by the addition of 500 µM IPTG and was carried out at 20°C overnight in the presence of 100 µM ZnCl<sub>2</sub> and 200 µM MgSO<sub>4</sub>. Bacteria were harvested by
centrifugation and resuspended in BugBuster® Protein Extraction Reagent (Millipore #70584-4) supplemented with protease inhibitor cocktail (Complete EDTA-free, Sigma-Aldrich).

Bacterial suspensions were incubated for 30 min at room temperature with 1 mg/ml lysozyme (Fluka) and further lysed by sonication. The soluble protein fraction was recovered by centrifugation. GST fusion proteins were isolated by binding to glutathione magnetic beads (MagneGST™ Glutathione Particles, Promega) for 30 min at room temperature on a rotating wheel. The beads were then washed three times with 0.65 M NaCl and three times with PBS.

ArcticExpress (DE3) competent cells (Agilent) were transformed with the expression vector pET-15-HIS-TRIM39 and pMAL-c2-TRIM17 (expressing MBP-TRIM17 fusion protein).

Bacteria were grown overnight in LB medium supplemented with Ampicillin and Gentamycin (20 µg/ml). Recombinant protein expression was induced by the addition of 1 mM IPTG and was carried out at 12°C overnight in the presence of 100 µM ZnCl₂ and 200 µM MgSO₄. Bacteria were harvested by centrifugation. Pellets were resuspended in bacterial lysis buffer (50 mM Tris- HCl [pH 8,6], 0.5 M NaCl, 50 mM MgSO₄) supplemented with lysozyme and protease inhibitors, and frozen in liquid nitrogen to lyse bacteria. Lysates were then cleared by centrifugation. MBP-TRIM17 proteins were purified on amylose resin (New England BioLabs, #E8021L) and then eluted in a buffer containing 20 mM maltose before dialysis in PBS. HIS-TRIM39 proteins were purified on Ni-NTA agarose beads (Qiagen, #1018244) and then eluted in a buffer containing 0.5 M imidazole before dialysis in PBS.

**In vitro ubiquitination assay**

NFATc3 was first transcribed and translated *in vitro*. For this, 1 µg of pCS2-HA-NFATc3 was incubated for 2 h at 30°C in 50 µl of the TNT® SP6 coupled wheat germ extract system (Promega, #L5030), according to the instructions of the manufacturer. For each ubiquitination condition, the equivalent of 3 µl of the *in vitro* translation reaction was immunopurified using 1 µl rat anti-HA antibody and 10 µl protein G-agarose beads in a buffer containing 50 mM Tris-HCl (pH 7.5) and 50 mM NaCl buffer for 1 h. Beads were washed 3 times in the same buffer, as described above for co-immunoprecipitation. Then, beads carrying NFATc3 were incubated in 20 µl of ubiquitination reaction buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 4 mM ATP, 4 mM MgCl₂, 2 mM DTT, 10 mM phosphocreatine, 0.5 U creatine kinase, 20 µM ZnCl₂), in the presence of 50 ng human recombinant His-tagged ubiquitin-activating enzyme E1 (from BostonBiochem, #E-304), 500 ng human recombinant His-tagged ubiquitin-conjugating
enzyme (E2) Ube2d3 (from BIOMOL International, #U0880), in the presence or the absence of 10 µg N-terminal-His-tagged ubiquitin (Sigma-Aldrich, #U5507), and ≈ 2 µg of purified recombinant mouse GST-Trim39 (WT) or GST-Trim39-C49S/C52S, or ≈ 2 µg of purified recombinant human His-TRIM39 in the presence or the absence of ≈ 2 µg MBP-TRIM17. Reactions were incubated at 37°C for 1 h. Beads were washed once and the reaction was stopped by adding 10 µl of 3 × Laemmli sample buffer and by heating at 95°C for 5 min. The samples were analyzed by SDS-PAGE and immunoblotting with anti-NFATc3, anti-Trim39 and anti-TRIM17 antibodies.

In vitro SUMOylation assay

WT NFATc3 and its KallR and EallA mutants were first transcribed and translated in vitro as described above. Equivalent amounts of the different forms of NFATc3 (between 1.5 and 6 µl of the in vitro translation reaction) were incubated for 1 h 30 min at 37°C in the presence of 3 µg recombinant SUMO1, 150 ng recombinant His-tagged Aos1/Uba2 (E1 enzyme), 100 ng recombinant Ubc9 (E2 enzyme) and 300 ng recombinant GST-PIASxα (E3 enzyme) in 20 µl shift-assay buffer (20 mM Hepes [pH 7.3], 110 mM KOAc, 2 mM Mg(OAc)₂, 0.5 mM EGTA, 1 mM DTT, 0.05% Tween 20, 0.2 mg/ml ovalbumin, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin) supplemented with 1 mM ATP. Recombinant proteins used in this assay were produced and purified as previously described (Bossis et al., 2005). Negative controls (time 0) were obtained by mixing all reagents directly into loading buffer. Reaction products were separated by SDS-PAGE (Tris-acetate gels), transferred to PVDF membranes and analyzed by western blot using anti-NFATc3 antibody.

RNA preparation and quantitative RT-PCR

Total RNA was extracted using the RNaseasy kit (Qiagen) and treated with the DNase I from the DNA-free™ kit (Thermo Fisher Scientific) according to manufacturer's instructions. RNA was used to perform a two-step reverse-transcription polymerase chain reaction (RT-PCR). In brief 1 µg of total RNA was reverse-transcribed using 200 U reverse transcriptase Superscript II (Thermo Fisher Scientific) in the presence of 2.5 µM N6 random primers and 0.5 mM dNTP. The equivalent of 6 ng of resulting cDNA was used as a template for real time PCR using a Mx3000P thermocycler (Agilent) with a home-made SYBR Green qPCR master mix (Lutfalla
PCR reactions were performed in 10 µl in the presence of 200 nM primers. Thermal cycling parameters were 10 min at 95°C, followed by 40 cycles of 95°C for 30 s, 64°C for 30 s and 72°C for 30 s. Specific primers used to amplify mouse Trim39 and mouse Trim17 are listed in supplementary Table 1. Data were analysed and relative amounts of specifically amplified cDNA were calculated with MxPro software (Agilent), using the mouse Gapdh amplicon as a reference.

Protein sequence analysis

The sequence of mouse Trim39 (GenBank: NM_024468) was analyzed by using the prediction web-based tools JASSA (Joined Advanced SUMOylation site and SIM analyzer, (http://www.jassa.fr/)) and GPS SUMO (group-based prediction system, http://sumosp.biocuckoo.org/online.php) to identify putative SUMO-interacting motifs.

Production of SUMO chains and GST-pull down

Recombinant free SUMO-2 and poly-SUMO-2 chains were produced in bacteria co-expressing His-SUMO-2, Aos1/Uba2 (SUMO conjugating E1 enzyme) and Ubc9 (SUMO E2 conjugating enzyme). For this, BL21 competent cells were transformed with the plasmid pE1-E2-His-Su2 (described in (Brockley et al., 2016)). The transformed bacteria were grown with strong agitation (210 rpm) at 37°C until the OD reaches 0.4-0.6. Protein expression was induced by adding 1 mM IPTG for 6 h at 25°C. The bacteria were resuspended in 30 ml of bacterial lysis buffer, frozen in liquid nitrogen and stored at -80°C. The resuspended bacteria were thawed and supplemented with lysozyme (1 mg/ml), 8 mM β-mercaptoethanol, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin and incubated for 1 h on ice before centrifugation at 100,000 x g for 1 h at 4°C. The supernatant was loaded on a Ni-NTA column equilibrated in bacterial lysis buffer supplemented with 8 mM β-mercaptoethanol, 0.5% Triton X-100, 10 mM imidazole and protease inhibitors. The column was washed 3 times with 10 ml of the same buffer and eluted with 15 ml of Ni-NTA elution buffer (bacterial lysis buffer supplemented with 250 mM imidazole and protease inhibitors).

For GST-pull down, GST-Trim39 and its different SIM mutants were produced in bacteria and purified as described above. The quantity and the quality of the different forms of GST-Trim39 bound to glutathione magnetic beads was first estimated on a poly-acrylamide gel using Coomassie blue staining, by reference to known amounts of BSA. Beads binding approximately
1 µg of each form of GST-Trim39 were incubated with ≈ 1 µg SUMO-2 chains for 1 h at room temperature in 200 µl shift assay buffer (20 mM Hepes [pH 7.3], 110 mM KOAc, 2 mM Mg(OAc)$_2$, 0.5 mM EGTA, 1 mM DTT, 0.05% Tween 20, 0.2 mg/ml ovalbumin, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin). Beads were recovered by centrifugation and washed 4 times with PBS. Material bound to the beads was eluted by the addition of 3 × Laemmli sample buffer and incubation at 95°C for 5 min. Both GST-Trim39 proteins and bound SUMO-2 chains were analyzed by immunoblotting.

Assessment of neuronal apoptosis

After 6 days in vitro (DIV), transfected or transduced CGNs were maintained in initial culture medium (control) or were washed once and incubated in serum-free BME supplemented with L-Gln, HEPES, antibiotics and 1 µM (+)-MK-801, and containing 5 mM KCl (K5 medium) for the indicated times. Neurons were then stained with DAPI and mounted on glass slides in Mowiol. In experiments in which the CGNs were transfected with GFP, GFP-NFATc3 or GFP-NFATc3-EallA, apoptosis was assessed among GFP-positive neurons, by examining neuronal morphology and nuclear condensation. For each experiment and each condition, at least 100 GFP-positive neurons were scored in a blinded manner. In experiments in which CGNs were transduced with shRNA-expressing lentiviruses, apoptosis was estimated by counting the percentage of condensed nuclei in five random fields for each condition (more than 500 cells).

Statistics

Statistical analyses were performed using GraphPad Prism version 7.0c for Mac OS X (GraphPad Software, San Diego California USA, www.graphpad.com). Unless stated, data are representative of at least three independent experiments.

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supported by COST (European Cooperation in Science and Technology). We would like to thank the staff of the Montpellier Genomic Collection platform for providing human TRIM39 and human TRIM17 cDNA clones. We acknowledge the imaging facility MRI (Montpellier Ressources Imagerie), member of the national infrastructure France-BioImaging infrastructure supported by the French National Research Agency (ANR-10-INBS-04, «Investments for the future». We are grateful to Frédérique Brockly for the production and purification of recombinant proteins. We thank Drs Dimitris Liakopoulos and Manuel Rodriguez for interesting discussions.

**Competing interests:** The authors declare that no competing interests exist.

**References**


Singh, S. K., Baumgart, S., Singh, G., Konig, A. O., Reutlinger, K., Hofbauer, L. C., Barth, P.,
Disruption of a nuclear NFATc2 protein stabilization loop confers breast and pancreatic cancer

Chung, E. J., Kim, J.-H., Chung, J.-Y., Hewitt, S. M., Baek, S., Lee, K.-M., Yee, C., Son, M.,
Multidrug Resistance and a Stem-like Phenotype in Immune Edited Tumor Cells. *Cancer Research, 77*(18), 5039–5053. https://doi.org/10.1158/0008-5472.CAN-17-0072


Hatakeyama, S. (2016). TRIM39 negatively regulates the NFκB-mediated signaling pathway

Terui, Y., Saad, N., Jia, S., McKeon, F., & Yuan, J. (2004). Dual role of sumoylation in the

Ulrich, J. D., Kim, M. S., Houlihan, P. R., Shutov, L. P., Mohapatra, D. P., Strack, S., &
Usachev, Y. M. (2012). Distinct activation properties of the nuclear factor of activated T-cells
(NFAT) isoforms NFATc3 and NFATc4 in neurons. *Journal of Biological Chemistry, 287*(45), 37594–37609. https://doi.org/10.1074/jbc.M112.365197

Urano, T., Usui, T., Takeda, S., Ikeda, K., Okada, A., Ishida, Y., Iwayanagi, T., Otomo, J.,
Ouchi, Y., & Inoue, S. (2009). TRIM44 interacts with and stabilizes terf, a TRIM ubiquitin E3

Uzunova, K., Götsche, K., Miteva, M., Weisshaar, S. R., Glanemann, C., Schnellhardt, M.,
Niessen, M., Scheel, H., Hofmann, K., Johnson, E. S., Praefcke, G. J. K., & Dohmen, R. J.

Nuclear factor of activated T-cells isoform c4 (NFATc4/NFAT3) as a mediator of antiapoptotic

*Cells, 8*(5). https://doi.org/10.3390/cells8050510

Vihma, H., & Timmusk, T. (2017). Sumoylation regulates the transcriptional activity of
https://doi.org/10.1016/j.neulet.2017.05.074

Disruption of PML nuclear bodies is mediated by ORF61 SUMO-interacting motifs and required for varicella-zoster virus pathogenesis in skin. *PLoS Pathogens*, 7(8), e1002157. https://doi.org/10.1371/journal.ppat.1002157


Figure 1. Trim39 interacts with both Trim17 and NFATc3.  
A. BHK cells were transfected with Trim17-GFP together with Flag-Trim39 or empty plasmid (as a negative control) for 24 h. Cells were then treated with 20 μM MG-132 for 5 h. The cells were subsequently harvested and lysates were subjected to immunoprecipitation using anti-Flag agarose beads (left panel) or GFP-Trap beads (right panel). Immunoprecipitates and total lysates were analyzed by western blot using anti-GFP and anti-Flag antibodies. 
B. Neuro2A cells were transfected with HA-NFATc3 together with Flag-Trim39 or empty plasmid for 24 h. Cells were then treated as in A and lysates were subjected to immunoprecipitation using anti-Flag (left panel) or anti-HA (right panel) antibodies. Immunoprecipitates and total lysates were analyzed by western blot using anti-HA and anti-Flag antibodies. 
C. Neuro2A cells were treated with 10 μM MG-132 for 4 h and then fixed and subjected to in situ PLA using rabbit anti-NFATc3 and mouse anti-Trim39 antibodies. Each bright red spot indicates that the two proteins are in close proximity. Negative control was obtained by omitting anti-Trim39 antibody. When indicated, cells were previously transfected with Trim39 for 24 h (overexpressed). Images were analyzed by confocal microscopy. To better visualize the differences in PLA intensity, maximum intensity projection was applied to the z-stacks of images on the left panel. To better determine the subcellular location of the NFATc3/Trim39 interaction, a single slice of the z-stack is presented on the right panel (endo 1 slice). Nuclear staining was performed using DAPI. 
D. Neuro2A cells were transduced with lentiviral particles expressing a control shRNA (sheGFP) or a specific shRNA against Trim39 (shTrim39#1) for 24 h. Transduced cells were selected using puromycin for two additional days and plated onto coverslips. The day after plating, cells were analyzed by immunofluorescence using two different antibodies against Trim39; in red: antibody from Origene, in green: antibody from Proteintech. Images were set to the same minimum and maximum intensity to allow signal intensity comparison. 
E. Additional coverslips from the experiment presented in D were treated as in C and z-stacks of images were subjected to maximum intensity projection.
Figure 2. Trim39 is an E3 ubiquitin-ligase of NFATc3. A. Neuro2A cells were transfected with HA-NFATc3 together with empty plasmid or His-tagged ubiquitin, in the presence or absence of Flag-Trim39 or the inactive mutant Flag-Trim39-DRING for 24 h. Cells were then incubated with 20 μM MG-132 for 6 h before harvesting. The ubiquitinated proteins were purified using nickel beads and analyzed by western blotting using anti-HA antibody to detect ubiquitin-conjugated HA-NFATc3. In a separate SDS-PAGE, samples of the input lysates used for the purification were analyzed with antibodies against HA and Flag. B. Neuro2A cells were transduced with lentiviruses expressing a control shRNA (directed against eGFP) or three different shRNAs targeting Trim39. Following 24 h transduction and 48 h selection of transduced cells using puromycin, cells were plated in new dishes. Then, cells were transfected with HA-NFATc3 or His-tagged ubiquitin or both for 24 h, and treated as in A. In conditions indicated by a star (*), some material was lost during TCA precipitation of the input lysate without affecting the amount of proteins in the nickel bead purification. These data are representative of 4 independent experiments. C. In vitro translated HA-NFATc3 was first immunopurified from wheat germ extract using anti-HA antibody. Then, beads used for immunopurification of NFATc3 were incubated for 1 h at 37°C in the in vitro ubiquitination reaction mix (containing E1 and E2 enzymes) with purified recombinant GST-Trim39 (WT) or its inactive mutant GST-Trim39-C49S/C52S (mut) in the presence or the absence of ubiquitin as indicated. Poly-ubiquitinated forms of NFATc3 were detected by immunoblotting using an anti-NFATc3 antibody. The same membrane was immunoblotted with an anti-TRIM39 antibody to verify that similar amounts of recombinant WT GST-Trim39 and GST-Trim39-C49S/C52S were used in the assay. Note that in the presence of ubiquitin the unmodified form of WT GST-Trim39 is lower due to high Trim39 ubiquitination.
Figure 3. Trim39 mediates NFATc3 degradation. A. BHK cells were transfected with a fixed amount of a HA-NFATc3 expressing vector (1 µg) together with empty plasmid (-) or increasing amounts of Flag-Trim39 expressing vector (0.1, 0.2, 0.5 and 1 µg) or 0.2 µg of a vector expressing the inactive mutant Flag-Trim39-ΔRING. When indicated, the cells were treated with 10 µM MG-132 for 6 h before harvesting. Total lysates were analyzed by western blot using antibodies against HA, Flag and actin. B. Neuro2A cells were left untreated (NT), or transfected twice with an siRNA targeting specifically Trim39 (siTrim39#1) or with a negative control siRNA (siCtrl) for 48 h. Total lysates were analyzed by western blot using antibodies against NFATc3, Trim39 and actin. The intensity of the NFATc3 bands on the western blots was quantified, normalized by the intensity of the actin bands and expressed relative to the values obtained with the control shRNA. The graph shows mean ± SEM from three independent experiments. **P<0.01 significantly different from siCtrl (one-way ANOVA followed by
Dunnett’s multiple comparisons test). C. Neuro2A cells were co-transfected with empty plasmid or HA-NFATc3, together with empty plasmid, Flag-Trim39 or the inactive mutant Flag-Trim39-ΔRING for 24 h. Then, total RNA was extracted and the mRNA level of Trim17 was estimated by quantitative PCR. Data are the means ± SEM of four independent experiments. **P<0.01 significantly different from the corresponding control (two-way ANOVA followed by Sidak’s multiple comparisons test). D. Neuro2A cells were transfected with empty plasmid, Flag-Trim39 or Flag-Trim39-ΔRING for 24 h. Then, cells were left untreated (control) or were deprived of serum for 3 h and subsequently treated with 1 μM A23187 and 100 nM PMA in serum-free medium for 1 h (A23+PMA). Total RNA was extracted and the mRNA level of Trim17 was estimated by quantitative PCR. Data are the means ± SEM of three independent experiments. *P<0.05; **P<0.01 significantly different from the corresponding value in cells transfected with empty plasmid (two-way ANOVA followed by Sidak’s multiple comparisons test). E. Neuro2A cells were transfected twice with two different siRNAs targeting specifically Trim39 or with a negative control siRNA for 48 h. Then, cells were left untreated (control) or were deprived of serum for 3 h and subsequently treated with 1 μM A23187 and 100 nM PMA in serum-free medium for 30 min (A23+PMA). Total RNA was extracted and the mRNA level of Trim39 (left panel) or Trim17 (right panel) was estimated by quantitative PCR (NT = non transfected). Data are the means ± SEM of six independent experiments. *P<0.05; ***P<0.001 significantly different from cells transfected with control siRNA in the same condition (two-way ANOVA followed by Sidak’s multiple comparisons test).
Figure 4. Trim17 inhibits TRIM39-mediated ubiquitination of NFATc3. A. BHK cells were transfected with HA-NFATc3 together with His-tagged ubiquitin, in the presence or the absence of Flag-Trim39, Trim17-GFP or both, as indicated, for 24 h. Then, cells were incubated with 20 µM MG-132 for 6 h before harvesting. The ubiquitinated proteins were purified using nickel beads and analyzed by western blotting using anti-HA and anti-Flag antibodies to detect poly-ubiquitinated forms of NFATc3 and Trim39. In a separate SDS-PAGE, samples of the input lysates used for the purification were analyzed with antibodies against HA, Flag and GFP. B. In vitro translated HA-NFATc3 was first immunopurified from wheat germ extract using anti-HA antibody. Then, beads used for immunopurification of NFATc3 were incubated for 1 h at 37°C in the in vitro ubiquitination reaction mix (containing ubiquitin and E1 and E2 enzymes) with purified recombinant His-TRIM39 or MBP-TRIM17 as indicated. Poly-ubiquitinated forms of NFATc3, TRIM39 and TRIM17 were detected by immunoblotting using anti-NFATc3, anti-TRIM39 and anti-TRIM17 antibodies revealed using high exposure times. Low exposure times were used to compare the level of TRIM39 and TRIM17 in the different conditions.
Figure 5. Trim17 reduces the interaction between endogenous Trim39 and NFATc3. A,B.
Neuro2A cells were transfected with HA-NFATc3 in the presence or the absence of Flag-
Trim39, Trim17-GFP or both, as indicated, for 24 h. Cells were then treated with 20 µM MG-
132 for 7 h. The cells were subsequently harvested and lysates were subjected to
immunoprecipitation using anti-HA (A) or anti-Flag (B) antibodies. Immunoprecipitates and
total lysates were analyzed by western blot using anti-HA, anti-GFP and anti-Flag antibodies.
The intensity of the bands containing Flag-Trim39 co-immunoprecipitated with HA-NFATc3
was normalized by the intensity of the bands corresponding to immunoprecipitated HA-
NFATc3 (A). The intensity of the bands containing HA-NFATc3 co-immunoprecipitated with
Flag-Trim39 was normalized by the intensity of the bands corresponding to

C

GFP

PLA

DAPI

D

number of dots/cell

0

5

10

15

20

25

GFP Trim17

-GFP

****
immunoprecipitated Flag-Trim39 (B). Relative values are indicated in red. C. Neuro2A cells were transfected with GFP or Trim17-GFP for 24 h. Then cells were treated with 10 µM MG-132 for 4 h, fixed and subjected to in situ PLA using rabbit anti-NFATc3 and mouse anti-Trim39 antibodies. Each bright red spot indicates that the two proteins are in close proximity. Images were analyzed by confocal microscopy and a single slice of the z-stacks is presented for each condition. Nuclear staining was performed using DAPI. Note that, in the Trim17-GFP condition, transfected cells (delineated by a yellow line) show less dots than neighboring non-transfected cells, which is not the case in the GFP condition. D. The number of dots was determined in individual cells transfected with either GFP or Trim17-GFP using Fiji. Data represent one experiment, including 68 transfected cells for each condition, representative of two independent experiments. ****p<0.0001, significantly different from GFP transfected cells (unpaired t test).
Figure 6. SUMOylation of NFATc3 modulates its ubiquitination and stability. A. In vitro translated HA-NFATc3 was incubated with in vitro SUMOylation reaction mix (containing SUMO1, E1, E2 and E3 enzymes) for 1 h 30 or directly added to sample loading buffer together with reaction mix (time 0). Multi-SUMOylated forms of NFATc3 were detected by immunoblotting using anti-NFATc3 antibody. B. Neuro2A cells were transfected with His-ubiquitin or empty plasmid, together with WT HA-NFATc3 or the different HA-NFATc3 E/A mutant constructs for 24 h. Then, cells were incubated with 20 µM MG-132 for 6 h before harvesting. The ubiquitinated proteins were purified using nickel beads and analyzed by western blotting using anti-HA antibody to detect ubiquitin-conjugated HA-NFATc3. In a separate SDS-PAGE, samples of the input lysates used for the purification were analyzed with...
antibodies against HA and tubulin. The intensity of the NFATc3 bands from the nickel bead purification was normalized by the intensity of the bands in the total lysates. Relative values are indicated in red. C. Neuro2A cells were transfected with WT HA-NFATc3 or NFATc3-EallA for 48 h. Then, cells were incubated with 20 µg/ml cycloheximide (CHX) for increasing times before harvesting. Proteins were analyzed by western blot using antibodies against HA tag and tubulin. D. The intensity of the bands on the western blots of different experiments performed as in C was quantified. For each experiment, the amount of NFATc3 was normalized by the level of tubulin in each condition and plotted against CHX incubation time. Data are the mean ± SEM of three independent experiments. ***p<0.0001, **p<0.005 significantly different from WT NFATc3 at the same incubation time (two-way ANOVA followed by Sidak’s multiple comparisons test).
Figure 7. Trim39 is a SUMO-targeted E3 ubiquitin-ligase for NFATc3. A. Neuro2A cells were transfected with His-tagged ubiquitin together with WT HA-NFATc3 or HA-NFATc3 EallA, in the presence or the absence of Flag-Trim39, for 24 h. Then, cells were incubated with 20 μM MG-132 for 6 h before harvesting. The ubiquitinated proteins were purified using nickel beads and analyzed by western blotting using anti-HA antibody to detect ubiquitin-conjugated HA-NFATc3. In a separate SDS-PAGE, samples of the input lysates used for the purification were analyzed with antibodies against HA and Flag. B. Neuro2A cells were transfected with Flag-Trim39 together with WT HA-NFATc3, HA-NFATc3-EallA or empty plasmid for 24 h. Cells were then treated with 10 μM MG-132 for 8 h. The cells were subsequently harvested and lysates were subjected to immunoprecipitation using anti-HA antibody (left panel) or anti-Flag beads (right panel). Immunoprecipitates and total lysates were analyzed by western blot using anti-HA and anti-Flag antibodies. The intensity of the bands containing Flag-Trim39 co-immunoprecipitated with HA-NFATc3 was normalized by the intensity of the bands of immunoprecipitated HA-NFATc3. The intensity of the bands containing HA-NFATc3 co-immunoprecipitated with Flag-Trim39 was normalized by the intensity of the bands of immunoprecipitated Flag-Trim39. Relative values are indicated in red. C. Recombinant GST, GST-Trim39 and its different SIM mutants were purified using glutathione beads and subsequently incubated with purified recombinant SUMO-2 and SUMO-2 chains. Material
bound to the beads was eluted and analyzed by western blot using anti-SUMO and anti-GST antibodies. A small fraction of the SUMO-2 chains was also loaded on the gel (input) for comparison. The intensity of bound SUMO-chain bands was quantified and normalized by the intensity of corresponding GST-Trim39 bands. Relative values are indicated in red. Note that SUMO bands are multiple of $\approx 15$ kDa corresponding to mono-, di-, tri-, tetra-SUMO etc…

D. Neuro2A cells were transfected with HA-NFATc3 or empty plasmid together with WT Flag-Trim39 or its SIM3 mutant for 24 h. Cells were treated as in B and lysates were subjected to immunoprecipitation using anti-HA antibody. Immunoprecipitates and total lysates were analyzed as in B. The intensity of the bands containing Flag-Trim39 co-immunoprecipitated with HA-NFATc3 was normalized by the intensity of the bands of immunoprecipitated HA-NFATc3. Relative values are indicated in red.

E. Neuro2A cells were transfected with His-tagged ubiquitin (or empty plasmid) together with HA-NFATc3 in the presence or the absence of Flag-Trim39 or its SIM3 mutant, for 24 h. Then, cells were treated as in A. Ubiquitinated proteins and input lysates were analyzed as in A. The intensity of the ubiquitinated forms of NFATc3 was quantified and normalized by the intensity of NFATc3 in the total lysate. Relative values are indicated in red.
Figure 8. SUMOylation and Trim39 attenuate NFATc3 pro-apoptotic effect in neurons.

A. CGN primary cultures were transfected after 5 days in vitro (DIV 5) with GFP (as a negative control), WT GFP-NFATc3 or GFP-NFATc3-EailA for 16 h. Then, neurons were switched to serum-free medium containing 5 mM KCl (K5) for 7 h or were left untreated (control). Following fixation, nuclei were visualized by DAPI staining and proteins fused to GFP were detected by fluorescent microscopy. Arrows indicate GFP-positive neurons with thick arrows for neurons undergoing apoptosis and thin arrows for healthy neurons. B. The percentage of transfected, GFP-positive neurons undergoing apoptosis was assessed by examining cell morphology and nuclear condensation. Data are the means ± S.E.M. of four independent experiments performed as in A. * P<0.05; *** * P<0.001 significantly different from the corresponding value obtained in neurons transfected with GFP (two-way ANOVA followed by Sidak’s multiple comparisons test). C. CGNs were transduced with lentiviral particles expressing a non-targeting control (directed against Luciferase) or an shRNA specifically targeting Trim39 one day after plating. At DIV 6, total cell extracts from KCl-deprived neurons were analyzed by western blot using anti-Trim39 antibody (Origene). D. CGN were transduced and treated as in C. At DIV 6 they were incubated for 8 h in K5 medium, fixed and stained with
The percentage of apoptotic neurons was estimated by examining nuclear condensation. Data are the means ± S.E.M. of four independent experiments performed as in D. **** $P<0.0001$ significantly different from neurons transduced with the control shRNA (two-way ANOVA followed by Sidak’s multiple comparisons test).
Supplemental data to:

Trim39 regulates neuronal apoptosis by acting as a SUMO-targeted E3 ubiquitin-ligase for the transcription factor NFATc3

by Meenakshi Basu Shrivastava, Barbara Mojsa, Stéphan Mora, Ian Robbins, Guillaume Bossis, Iréna Lassot and Solange Desagher

**Figure S1**

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**B**

![B](image)
Figure S1. Trim39 does not alter calcium-induced nuclear translocation of NFATc3. A. BHK cells were transfected with HA-NFATc3 together with GFP (negative control) or Trim17-GFP, or Trim39-mCherry for 24 h. Then, cells were left untreated (control) or deprived of serum for 3h and treated with 1 µM of the calcium ionophore A23187 in serum-free medium for an additional 30 min before fixation. NFATc3 was detected using an anti-HA antibody and visualized by confocal microscopy. GFP, Trim17-GFP and Trim39-mCherry were visualized by GFP or mCherry fluorescence and nuclei were stained with DAPI. B. Quantification of the nuclear localization of NFATc3 in experiments conducted as in A. The percentage of cells showing NFATc3 mainly in the nucleus was determined among the population of cells expressing both HA-NFATc3 and either GFP, Trim17-GFP or Trim39-mCherry. Data are the means ± SD of three independent experiments. **** P<0.0001 significantly different from the corresponding value obtained in cells transfected with GFP and NFATc3 (two way ANOVA followed by Dunnett’s multiple comparisons test).

Figure S2. Trim39 decreases the ubiquitination of Trim17. The PVDF membrane presented in Figure 4A was stripped and blotted with an anti-GFP antibody.

Figure S2. Trim39 decreases the ubiquitination of Trim17. The PVDF membrane presented in Figure 4A was stripped and blotted with an anti-GFP antibody.
Figure S3. SIM3 is the predominant SIM involved in the interaction of Trim39 with SUMO-2 chains. Recombinant GST, GST-Trim39 and its different double or triple SIM mutants were purified using glutathione beads and subsequently incubated with purified recombinant SUMO-2 and SUMO-2 chains. Material bound to the beads was eluted and analyzed by western blot using anti-SUMO and anti-GST antibodies. A small fraction of the SUMO-2 chains was also loaded on the gel (input) for comparison. The intensity of bound SUMO-chain bands was quantified and normalized by the intensity of corresponding GST-Trim39 bands. Relative values are indicated in red.
Supplementary Table 1: list of the primers used to generate described constructs

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<tr>
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Unpublished Results

NFATc3

Challenges
As mentioned before, Barbara had already shown that Trim39 interacts with NFATc3 and induces the ubiquitination of NFATc3. However, towards the end, she had problems with the expression of NFATc3. Therefore, when I arrived, in order to get high expression of NFATc3, I cloned NFATc3 into an expression vector driven by a stronger promoter (human cytomegalovirus major immediate early gene enhancer/promoter region). This was really critical as the few experiments which I had performed with the old construct failed to express NFATc3. Furthermore, I had observed in several experiments that NFATc3 is usually more prone to degradation in cell lysates. Therefore, I had to optimize the conditions for the co-immunoprecipitation and \textit{in-vivo} ubiquitination experiments of NFATc3.

I also attempted to examine whether overexpression or silencing of Trim39 could impact the stability of either over-expressed or endogenous NFATc3. For this, I performed a series of experiments whereby I co-transfected Trim39 and NFATc3 in Neuro2A cells and treated the cells with a protein synthesis inhibitor, cycloheximide. Unfortunately, the results from such experiments were not conclusive as the half-life of Trim39 is not longer than that of NFATc3 and cycloheximide prevents the expression of both the proteins. Using an alternate approach, I then silenced Trim39 either by using siRNAs or shRNA’s to examine its effect on the half-life of endogenous NFATc3. I again performed series of experiments in Neuro2A cells treated with cycloheximide and I measured the level of endogenous NFATc3. However, sometimes the silencing of Trim39 was not efficient and moreover, we did not have a good antibody against NFATc3 at the time. In parallel, using wheat germ extracts, I produced NFATc3 using \textit{in vitro} transcription/translation and attempted to perform \textit{in-vitro} ubiquitination and \textit{in-vitro} SUMOylation experiments. However, in my hands, NFATc3 was much degraded and even after optimization, I could not succeed in such experiments. Hence, I am really grateful to Irina Lassot for her expertise in performing the \textit{in-vitro} experiments pertaining to NFATc3 in this study.

Results

\textit{Trim39 is a SUMO-targeted E3 ubiquitin-ligase}

SUMO interacting motifs (SIMs) facilitate the recognition of SUMO-modified proteins which may trigger different cellular responses. I had found three SIM sites in Trim39 [SIM1: PVII, SIM2: VCLI, and SIM3: LLSRL] located in the RING domain, B-Box domain, and the Coiled-Coil domain,
respectively. Indeed, the mutation of SIM3 (LLSRL → AAARA) resulted in a strong reduction in its ability to bind SUMO2 chains. To confirm, these results, I further tested the ability of Trim39 to bind tetra-SUMO2 fusion protein that contains four SUMO-2 molecules (4xS2—a kind gift from Dr. Manual Rodriguez). Using GST pulldown assay, I found that while GST alone showed no binding, GST-Trim39 could clearly bind to 4xS2. Strikingly again, mutation of SIM3 showed a strong reduction in the binding of 4xS2 (Figure 52). In conclusion, these results further confirm first, my findings on the ability of Trim39 to bind SUMO2 chains and second, that indeed SIM3 of Trim39 plays a critical role in its binding.

NFATc4

Introduction

As previously mentioned, NFATc4 is a member of NFAT family and alongside NFATc3, it is also highly expressed in neurons (Ulrich et al. 2012; Vihma et al. 2016a). In a previous study, it was shown that Trim17 could interact with NFATc4, however, this interaction was SUMOylation-independent. Additionally, it was also shown that Trim17 binds to NFATc4 and inhibits its nuclear-translocation (Mojsa et al. 2015a). When I arrived in the group, Barbara had preliminary results suggesting that NFATc4 was also a substrate of Trim39. These results included in vivo ubiquitination of NFATc4 both in the presence and absence of Trim39 and Trim17, and after silencing of endogenous Trim39. I then confirmed the interaction of NFATc4 and Trim39 by co-immunoprecipitation, effect of Trim39 on the protein level of NFATc4, in vivo ubiquitination of
NFATc4 mediated by Trim39 but not by an inactive mutant. Furthermore, I examined the inhibitory effect of Trim17 on the NFATc4/Trim39 interaction. However, during the study, as I decided to develop the part on Trim39 being a STUbL of NFATc3 and I had to optimize many experimental conditions for that, we decided to not pursue the part on NFATc4. Furthermore, as SUMOylation did not seem to be important for the interaction of NFATc4 and Trim17, probably because NFATc4 has a short C-Terminal region, we decided to focus only on NFATc3. However, the few results obtained for NFATc4 described below are indeed very promising.

**Results**

**Trim39 interacts with NFATc4**

To confirm that NFATc4 and Trim39 interact with each other, I performed co-immunoprecipitation experiment after co-transfection of NFATc4 and Trim39 in Neuro2A cells. Indeed, NFATc4 and Trim39 were found to reciprocally co-immunoprecipitate with each other using anti-Flag and anti-HA antibodies (Figure 53). However, these results need to be further confirmed by experiments such as *in situ* proximity ligation assay to confirm interactions between endogenous proteins.

**Trim17 inhibits the interaction between Trim39 and NFATc4**

Barbara had observed that Trim17 does not induce ubiquitination of NFATc4 and the presence of Trim17 inhibits the ubiquitination of NFATc4 induced by Trim39. Therefore, to understand the
mechanism underlying this inhibitory effect of Trim17, we tested whether this was due to the inhibition of the interaction between NFATc4 and Trim39, in line with our observation with NFATc3, Trim17, and Trim39. Similar to our results for NFATc3, we could observe a strong interaction between NFATc4 and Trim39, however, in the presence of Trim17 the interaction between Trim39 and NFATc4 was drastically reduced (Figure 54). Altogether, these preliminary results suggest that Trim17 inhibits the ubiquitination of NFATc4 by Trim39, partly, by preventing the interaction between NFATc4 and Trim39.

**Conclusion**

Taken together our results suggest that Trim39 is also an E3 ubiquitin-ligase of NFATc4. Indeed, Trim39 interacts with NFATc4 and also induces its ubiquitination (Barbara Mojsa, unpublished results). In contrast, silencing endogenous Trim39 drastically abolishes the ubiquitination of NFATc4 (Barbara Mojsa, unpublished results), further supporting that it is a major E3 ubiquitin-ligase regulating NFATc4. Furthermore, similar to NFATc3, we also observed the same inhibitory effect of Trim17 on the Trim39 mediated ubiquitination of NFATc4 (Barbara Mojsa, unpublished results), which could be due to inhibition of the interaction between NFATc4 and Trim39 by Trim17. However, these results need to be supported by additional experiments. Nevertheless, our results suggest that NFATc4 is another substrate for Trim39.

Figure 54: Trim17 reduces the interaction between Trim39 and NFATc4. N2A cells were transfected with HA-NFATc4 along with Flag-Trim39, Trim17-GFP or empty plasmid (as a negative control) for 24h. Following treatment with MG-132 for 5h, cells were harvested and lysates were subjected to immunoprecipitation using anti-Flag agarose beads (left) or anti-HA agarose beads (right) (Data representative of 4 experiments).
General Discussion

1) Trim39 is a genuine E3-ubiquitin ligase of NFATc3 that regulates its degradation

NFATc3 has been shown to play critical roles in the nervous system and disease. Two studies have shown that the calcineurin/NFATc3 signalling pathway in mouse dopaminergic neurons (mDA) might be implicated in PD (J. Luo et al. 2014; Caraveo et al. 2014). Indeed, it was found that the neurotoxic effects of α-synuclein might be mediated by NFATc3 (J. Luo et al. 2014). My group had also shown that NFATc3 aggravated neuronal death induced by survival-factor deprivation whereas NFATc4 was neuro-protective (Mojsa et al. 2015b). The fact that NFATc3 plays an important role in neuronal apoptosis is further supported by studies from other groups (Jayanthi et al. 2005; Gómez-Sintes and Lucas 2010). Even though NFATc3 is important for various cellular functions and is deregulated in diseases, the regulation of its protein stability had been poorly studied. NFAT are relatively short-lived proteins and understanding the mechanisms by which their stability is regulated is extremely critical to control their activity. Notably, no formal E3 ubiquitin-ligase for NFATc3 had been identified.

In this study, we have shown that Trim39 and NFATc3 interact with each other. This interaction was first confirmed using co-immunoprecipitation between overexpressed NFATc3 and Trim39. Importantly, we have also shown the interaction between endogenous NFATc3 and Trim39 using in situ proximity ligation assay (PLA) which takes place predominantly in the cytoplasm. Further, overexpression of Trim39 induced the ubiquitination of NFATc3 whereas the dominant negative mutant Trim39ΔRING did not have the same effect. This confirmed that the RING domain of Trim39, that is responsible for its E3 ubiquitin-ligase activity, is involved in the ubiquitination of NFATc3. Moreover, our study shows that NFATc3 is a direct substrate of Trim39 as our in vitro ubiquitination data confirmed that Trim39 directly induces the ubiquitination of NFATc3. We also found that Trim39 impacts the protein levels of NFATc3. Overexpression of Trim39 decreased the protein level of NFATc3 in a dose-dependent manner whereas silencing of Trim39 increased the protein level. Therefore, our results strongly support that Trim39 is an E3 ubiquitin-ligase of NFATc3 that favours its degradation.

Interestingly, our study further raises other questions such as first, is Trim39 the only E3 ubiquitin-ligase of NFATc3? To address this question, the phrase ‘One E3-one substrate-one function’ is not true (Napolitano and Meroni 2012b). It has been observed for many proteins that there are more than one E3 ubiquitin-ligase which regulate them. For instance, there are many known E3 ubiquitin-ligases for the tumor suppressor p53 such as murine double minute 2 (MDM2) and Trim39 to name a few. Despite the significance of MDM2 in the regulation of p53 levels, p53 was still found to be susceptible to degradation in MDM2-deficient mice. Zhang et al., 2012 identified that Trim39 was also a direct E3 ubiquitin-ligase of p53 in the context of cancer (Liguo Zhang et al. 2012b). Moreover, a recent study by Chao et al., 2019 suggested that CHIP might be a plausible E3 ubiquitin-ligase of NFATc3 in LPS-induced cardiomyopathies (Chao et al. 2019), however, in this study there are
several key experiments which are missing to really demonstrate that CHIP is a genuine E3 ubiquitin-ligase of NFATc3. Through our study, we have identified Trim39 as a genuine and direct E3 ubiquitin-ligase of NFATc3, however, we cannot exclude the possibility that there might be other E3 ubiquitin-ligase which regulate NFATc3 stability.

The second question is besides NFATc3, does Trim39 target any other substrate for ubiquitin-mediated proteasomal degradation? Our preliminary results on NFATc4 suggest that overexpression or silencing of Trim39 either increases or decreases the ubiquitination of NFATc4, respectively. This suggests that Trim39 also targets NFATc4 for ubiquitination, however, these results need to be further validated. Additionally, a study done by Fan et al., 2008 showed that NFATc4 was ubiquitinated via Lysine48-linked polyubiquitin chains and this modification further decreased its protein levels in H9c2 cardiac cells. Based on our preliminary results on NFATc4, it is tempting to speculate that Trim39, which is also expressed in the heart, might regulate the stability of NFATc4 in cardiac development and hypertrophy (Y. Fan et al. 2008b).

2) The E3 ubiquitin ligases Trim17 and Trim39 have antagonistic roles in the regulation of NFATc3

In the present study, using co-immunoprecipitation, we have shown that Trim17 and Trim39 strongly interact with each other. Interestingly, Trim17 is not an E3 ligase of NFATc3 whereas Trim39 ubiquitinates and degrades NFATc3. Two studies in my group had shown that TRIM17 interacts with other TRIM partners and inhibits their E3 ubiquitin-ligase activity (Iréna Lassot et al. 2018; Lionnard et al. 2019). Strikingly, our in-vivo ubiquitination results suggest that Trim17 inhibits the ubiquitination of NFATc3 mediated by Trim39. This inhibitory effect of Trim17 on the E3 ubiquitin-ligase activity of Trim39 was also confirmed in-vitro in a cell free system suggesting that indeed this is a direct effect. There could be several mechanisms by which Trim17 inhibits Trim39-mediated ubiquitination of NFATc3. First, Trim17 may directly inhibit the interaction between Trim39 and NFATc3. Indeed, this is supported by series of experiments. Using, co-immunoprecipitation, we have shown that Trim39 and NFATc3 strongly interact with each other, however, this interaction is strongly abrogated in the presence of Trim17. This was also confirmed using reverse co-immunoprecipitation. Importantly, our PLA results have confirmed that compared to the non-transfected cells, when we overexpress Trim17, there is a drastic reduction in the interaction between endogenous Trim39 and NFATc3.

A second mechanism could be that Trim17 directly inhibits the E3 ubiquitin-ligase activity of Trim39. Indeed, several studies have shown that homo-oligomerisation of TRIM proteins is a prerequisite to be an active E3 ubiquitin-ligase (Streich et al. 2013; Koliopoulos et al. 2016). Indeed, a direct inability of Trim39 to auto-ubiquitinate was confirmed in our in vitro experiments in the presence of Trim17. These results suggest that Trim17 directly inhibited the auto-ubiquitination of Trim39. Altogether, it might be possible that Trim39 forms inactive hetero-oligomers with Trim17 at the expense of its E3-ubiquitin ligase activity. Furthermore, auto-ubiquitination is a characteristic feature of RING domain E3 ligases and it is the auto-ubiquitination which can be used to monitor the E3 ligase activity in the absence of a specific target (Yili Yang and Yu 2003; Lamothe et al. 2007).
This mechanism where Trim17 inhibits the interaction between a substrate and its E3 ubiquitin-ligase and antagonistically regulate a protein is not an isolated event as it has also been shown in two studies in my group. For instance, TRIM17 has been shown to inhibit TRIM41-mediated ubiquitination of ZSCAN21 and TRIM28-mediated ubiquitination of BCL2A-1 (Iréna Lassot et al. 2018; Lionnard et al. 2019). Additionally, our preliminary results on NFATc4 suggests that Trim17 stabilizes NFATc4 and inhibits the ubiquitination of NFATc4 mediated by Trim39 possibly by preventing the interaction between NFATc4 and Trim39. However, these results need further validation. Importantly, these two mechanisms are not mutually exclusive as the formation of the hetero-oligomers between Trim17 and Trim39 might prevent the binding of Trim39 to NFATc3. Furthermore, it is also possible that Trim17 and Trim39 compete with each other for NFATc3 binding. Altogether, our results strongly suggest that Trim17 stabilizes NFATc3 by inhibiting Trim39-mediated ubiquitination of NFATc3, partly, by preventing the interaction between NFATc3 and Trim39.

Another question arises that arises is why Trim17, which was identified as an E3 ubiquitin-ligase, does not induce the ubiquitination and degradation of NFATc3 but rather stabilizes it? It might be possible that the stability of NFATc3 by Trim17 is context and substrate-dependent. The scenario where a known E3 ubiquitin-ligase stabilizes a protein by inhibiting its ubiquitination and degradation has been shown previously. Trim39, for instance, is known to prevent the ubiquitination and degradation of MOAP-1, and p21 by inhibiting their E3 ubiquitin-ligases, APC/C Cdh1 and CRL4Cd2, respectively (N.-J. Huang et al. 2012b; Lei Zhang et al. 2012b).

Moreover, it is fairly possible to have an E3-substrate relationship between two E3 ubiquitin-ligases as observed between Trim21 and Trim5 where the former ubiquitinates and degrades the latter (Napolitano and Meroni 2012b). However, it was interesting to observe in our experiments that Trim17 and Trim39 did not ubiquitinate and induce the degradation of each other as evident in the input of co-immunoprecipitation experiments and also that in each other’s presence, the ubiquitination of both Trim17 and Trim39 did not increase (both in vivo and in vitro).

### 3) NFATc3 at the center of the crosstalk between ubiquitination and SUMOylation

Since the discovery of SUMOylation more than 20 years ago, it has been critically involved in various cellular and biological processes such as regulation of transcription, cell cycle progression, maintenance of genome integrity, protein-protein interactions, intracellular transport, protein stability, etc. (Hay 2005; Geoffroy and Hay 2009; M. H. Tatham et al. 2011). In the previous study, my group had shown that NFATc3 had three conserved SUMOylation sites at K435, K704, and K1013 and that the sites K704 and K1013 were the main SUMOylation sites in NFATc3 (Mojsa et al. 2015b). It is known that the same K residue could be either ubiquitinated or SUMOylated (J. M. Desterro, Rodriguez, and Hay 1998; Geoffroy and Hay 2009; Liebelt and Vertegaal 2016; S. K. Singh et al. 2011). Therefore, Barbara Mojsa generated E/A mutants of NFATc3 where the second conserved glutamate residue (E) was mutated to Alanine (A) which is known to disrupt the SUMO conjugation. This enabled us to prevent SUMOylation without preventing NFATc3’s ubiquitination on the K residues of these sites. Interestingly, in the present study, our in vitro data show that compared to the WT-NFATc3, SUMOylation of the EallA mutant of NFATc3 (where all the three conserved glutamate residues have been mutated to alanine) and the classical KallR mutant of
NFATc3 (all conserved K residues mutated to R) has been abolished, indicating that these sites of NFATc3 are important for its SUMOylation. Furthermore, the initial understanding that SUMO does not target proteins for degradation has been revisited long ago. Studies have shown that the SUMOylated proteins could also be proteolytically downregulated by the UPS (Uzunova et al. 2007; Miteva et al. 2013). Importantly, studies in yeast as well as in human cells support that SUMO can act as a signal to ubiquitinate and degrade the modified protein confirming a crosstalk between the two pathways (Geoffroy and Hay 2009). In line with these observations, our in vivo ubiquitination data indicates that the EallA mutant of NFATc3 was less ubiquitinated than the WT-NFATc3 and other NFATc3-E/A mutants, clearly suggesting that SUMOylation sites of NFATc3 are important for its ubiquitination. Importantly, our results also show that the half-life of NFATc3-EallA mutant is increased significantly compared to the WT-NFATc3 after inhibition of protein synthesis with cycloheximide. Altogether, our data suggest that SUMOylation of NFATc3 acts as a signal for ubiquitin-mediated proteasomal degradation and implicates SUMOylation as a requirement for subsequent ubiquitination.

This is really interesting as SUMO and ubiquitin are known to play antagonistic roles, however, in the case of NFATc3, SUMOylation and ubiquitination work in synergy to promote its ubiquitination and proteasomal degradation. SUMOylation of other NFAT members have also been reported. For instance, the study by Terui et al., showed that the K residues K684 and K897 in NFATc2 are target sites for SUMOylation (Terui et al. 2004) and interestingly, Singh et al. 2011, had shown that K684 and K897 were less ubiquitinated than the WT-NFATc2 (S. K. Singh et al. 2011). Therefore, it might be possible that SUMOylation of NFATc2 on these residues might be important to favour the ubiquitination and proteasomal degradation of NFATc2 as well. Additionally, a similar interplay between ubiquitination and SUMOylation has also been observed for another transcription factor, PEA3. Guo et al. 2011 showed that the PEA3-E12345A mutant (where all the conserved glutamic acid residues were mutated to A) was less ubiquitinated than the WT-PEA3. Moreover, they also showed that SUMOylation of PEA3 is important for its degradation further supporting that SUMOylation promotes ubiquitination and degradation of proteins (B. Guo et al. 2011).

4) Characterization of Trim39 as a SUMO Targeted Ubiquitin Ligase (STUbL) for NFATc3

In the present study, we have confirmed that Trim39 is an E3 ubiquitin-ligase of NFATc3. As NFATc3 had been shown to be SUMOylated, we found that compared to the WT-NFATc3, the mutant NFATc3-EallA is less ubiquitinated by Trim39. These results suggest that Trim39 preferentially ubiquinates the SUMOylated forms of NFATc3. Furthermore, we have shown that Trim39 interacts strongly with the WT-NFATc3, however, its interaction with NFATc3-EallA mutant decreases sharply. This data suggests that SUMOylation sites in NFATc3 are important for its interaction with Trim39. These results encouraged us to examine whether Trim39 could be a SUMO targeted E3 Ubiquitin-ligase for NFATc3.

STUbLs are proteins that preferentially target SUMO-modified proteins for ubiquitin-mediated proteolysis. They are characterized by the presence of a RING domain and SUMO Interaction Motifs (SIMs). The RING domain mediates the E3 ubiquitin-ligase activity while SIMs facilitate the recognition of SUMO. As mentioned earlier, SIMs are classified into SIMa (four consecutive
hydrophobic residues followed by a mixed cluster of S/D/E residues), SIMr (four hydrophobic acids preceded by an acidic cluster), and SIMb (V-I-D-L-T, with some variability in the first two hydrophobic positions, followed by a stretch of S/E/D regions). However, there are also putative SIMs which consist of four hydrophobic residues flanked by acidic residues on both sides, hence, precluding a classification of either SIMa or SIMr type (Miteva et al. 2013). Furthermore, it is not clear if the class of SIMs have a major influence on their recognition properties. So far, only two human STUbLs namely RNF4 (Michael H. Tatham et al. 2008) and RNF111/Arkadia (Erker et al. 2013) have been characterized, however, considering the presence of large number of SUMOylated proteins, there might be certainly other STUbLs controlling their regulation, which have not yet been identified.

In order to examine the possibility that Trim39 is a STUbL, we first searched for putative SIMs in Trim39. Using a combination of two different softwares, JASSA (Beauclair et al. 2015) and GPS-SUMO (Q. Zhao et al. 2014), we identified three putative SIMs in Trim39. These SIMs were chosen on the basis of their score which was awarded based on the stringency condition of ‘Medium SUMO Interaction Threshold’. SIM1 was located in the RING domain, SIM2 in B-Box domain, and SIM3 in the coiled coil domain. The 3D structure of the RING domain and B-Box domain of TRIM39 suggests that the SIMs 1 and 2 are exposed and accessible. Unfortunately, the structure of the coiled coil domain is not yet available. Interestingly, unlike RNF4 and RNF111, the two of the three putative SIMs in Trim39 namely, SIM1 (PVII) and SIM2 (VCLI), do not necessarily conform to the three major SIM types classified (SIMa, SIMr, and SIMb). However, they do fit the best-studied SIMs which contain three hydrophobic residues (typically valine, leucine or isoleucine) in a sequence of four amino acids (V/L/I, X, V/L/I, V/L/I) flanked by acidic amino acid residues (E and D) on both the sides. Importantly, the putative SIMs in Trim39 do not conform to the typical SIMs described (Figure 55).

Using GST-pulldown with GST-WT-Trim39 and GST-Trim39 SIM mutants, I confirmed that Trim39 binds to SUMO2 chains and particularly, to the -di, -tri, -polymeric forms of SUMO2 chains but not to the free SUMO. Notably, the mutation of SIM1 and SIM2 did not significantly affect the binding of Trim39 to SUMO2 chains, however, mutation in the third SIM of Trim39 (LLSRL), strongly reduced its ability to bind SUMO2 chains (a decrease of about 80% observed compared to the WT-Trim39). This suggests that SIM3 alone is important for SUMO binding and notably, we can observe a drastic decrease in its binding to -di and -poly SUMO polymers. The significance of SIM3

<table>
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Figure 55: Three putative SIM sites in Trim39 obtained by using GPS-SUMO (confirmed using JASSA).
in Trim39 was also confirmed by using tetra-SUMO chains (unpublished results) where we could observe a similar decrease in SIM3 binding to 4x-SUMO compared to WT-Trim39. In order to establish the role of the SIMs in Trim39, we examined the ability of its SIM mutants to interact with and ubiquitinate its substrate NFATc3. Indeed, our co-immunoprecipitation and ubiquitination experiments suggest that SIM3 in Trim39 is important to mediate its interaction with and ubiquitination of NFATc3. We also observed that all the SIM mutants of Trim39 were competent for auto-ubiquitination (data not shown).

In conclusion, our results show that Trim39 ubiquititates the SUMOylated forms of NFATc3 and that the SUMOylation sites of NFATc3 are important for its interaction with Trim39. Furthermore, SIM3 in Trim39 is important for poly-SUMO-2 binding, interaction, and ubiquitination of NFATc3. Taken together, our results characterize Trim39 as a STUbL for NFATc3.

In the STUbL Arkadia, it was shown that mutation of the third SIM alone most strongly decreased its binding to SUMO-1 and SUMO-3 whereas mutation of the three SIM sites was necessary to observe a complete abrogation of its binding to SUMO-1 and SUMO-3. With Trim39, it was interesting to observe that similar to Arkadia, mutation in SIM3 alone had a drastic effect in its binding to SUMO-2 chains, its interaction with NFATc3, and its ability to decrease the ubiquitination of NFATc3. Strikingly, the mutation in all the three SIM sites decreased its interaction with NFATc3, however, did not completely abrogate it. This might be due to the presence of other unverified SIMs in Trim39. It is important to note that while searching for putative SIM sites in Trim39, we could also observe the presence of eight other SIMs with a score ranging between 36 to 50, that are also conserved in human Trim39. Another explanation could be that there might be some non-canonical SIMs in Trim39 (Kerscher 2007). Moreover, the characterization of Trim39 as a STUbL is an example of transition from bioinformatics prediction to experimental validation and opens the possibility to explore the existence of other STUbLs.

In the two mammalian STUbLs identified so far, it was observed that the presence of closely spaced multiple SIMs is required for efficient binding to SUMO. In Trim39, in contrast, the three SIMs are quite distant to one another and only one SIM plays an important role in the binding, ubiquitination and interaction with NFATc3. The third SIM is located in the coiled-coil domain of Trim39 and coiled-coil domains in TRIM proteins are both necessary and sufficient for protein homo or multi-dimerization. Therefore, the importance of SIM3 for binding to SUMO might be related to the ability of Trim39 to form homo-dimers. This homo-dimerization of Trim39 might place the two coiled-coil domains of Trim39 in an anti-parallel orientation (as it has been observed for other TRIM proteins) (Esposito, Koliopoulos, and Rittinger 2017), which may bring two SIM3 in close proximity and favour the interaction with SUMO chains. Moreover, this dimeric or multi-oligomeric structural arrangement of TRIM proteins brings 2 or more RING domains, B-Box domains, PRY-SPRY domains in close-proximity. Therefore, despite being distantly spaced, the SIMs in Trim39 might be able to efficiently bind SUMO chains. More insights could be possibly gained through structural studies which would need a prediction for the coiled-coil domain structure of Trim39. Moreover, the recognition of SUMO through a single SIM has been reported for other proteins. For instance, the SUMOylated form of PCNA (proliferating cell nuclear antigen) had been identified as a physiological substrate for Rad18, a RING finger ubiquitin ligase in S. cerevisiae. The binding of SUMO to Rad18 is also mediated by a single SIM (Parker and Ulrich 2012). Finally, the ability of
proteins with a single SIM to efficiently bind poly-SUMO chains has been observed in the case of Zip1 in *S. Cerevisiae* (C.-H. Cheng et al. 2006b) and CENP-E (centromere-associated protein-E - a microtubule motor protein which binds preferentially to SUMO-2 chains compared to SUMO-1) (X.-D. Zhang et al. 2008). Although they are not STUbLs, both these proteins have a preference for binding to poly-SUMO chains via a single SIM, which further supports that one SIM alone can mediate the binding to poly-SUMO2 chains.

5) **Functional impact of Trim39 and SUMOylation on NFATc3**

Both SUMOylation and members of TRIM family are critically involved in the regulation of key cellular processes such as regulation of cell cycle, differentiation, development, apoptosis, transcription to name a few. In this study, we have focused on the participation of Trim39 and SUMO in regulating two cellular processes: transcription and apoptosis.

**Transcription**

In the present study, we examined whether the effect of Trim39 on the protein level of NFATc3 could have an impact on its transcriptional activity measured by the mRNA level of one of its target gene: *Trim17*. Indeed, our data shows that the co-transfection of Trim39 significantly reduced the increase in the mRNA level of *Trim17*, mediated by overexpressed NFATc3. In contrast, silencing endogenous Trim39 increased the expression of Trim17. Furthermore, after treatment with the calcium ionophore A23184 and with the Phorbol-myristate acetate (PMA) (known to activate endogenous NFAT proteins and their partners such as AP-1), overexpression of Trim39 decreased the mRNA levels of Trim17 whereas silencing Trim39 had an opposite effect. This increase in Trim17 expression is certainly due to endogenous NFATc3 as it is the most abundant and expressed transcription factor in various neuronal cell types (Ulrich et al. 2012; Vihma et al. 2016a; Vihma and Timmusk 2017) and also in Neuro 2A cells (Mojsa et al. 2015b). As Trim17 had been found to inhibit NFATc3 by retarding it in the cytoplasm (Mojsa et al. 2015b), we verified that Trim39 had no effect on the nuclear-translocation of NFATc3. Altogether, these data suggest that Trim39 inhibits the transcriptional activity of NFATc3 by ubiquitin-dependent proteasomal degradation without affecting its nuclear translocation.

TRIM E3 ubiquitin-ligases have been shown to target different transcription factors for ubiquitin-dependent proteasomal degradation, consequently, controlling their transcriptional activity. For instance, my group has shown that Trim17 binds to NFATc3 and consequently the transcriptional activity of NFATc3. In another study, my group had also shown that the E3 ubiquitin-ligase TRIM41 induces the ubiquitination and the degradation of transcription factor ZSCAN21, thereby reducing the expression of its target gene *SNCA* (Irénä Lassot et al. 2018). Furthermore, other TRIM E3 ubiquitin-ligases such as RET finger protein (RFP) and TIF1 (Transcriptional intermediary factor 1) regulators have been shown to target other transcription factors (Cammas et al. 2012b).

Due to time constraints, our study on the impact of Trim39 on the transcriptional activity of NFATc3 could not address few questions. For instance, SUMOylation has been shown to play crucial roles in nuclear translocation and transcriptional regulation of different proteins including NFAT (Nayak et
al. 2009; Li Chen et al. 2013; Sun et al. 2014; Vihma and Timmusk 2017). Therefore, it is possible that the transcriptional activity of NFATc3 might be regulated by its SUMOylation. SUMOylation of a transcription factor generally causes a reduction in its activity or an increase in its repressive activity associated with the transcription factor (Gill 2003). It could therefore be interesting to examine whether the SUMOylation affects the activity of NFATc3. To establish this, we could compare the activity of WT-NFATc3 with the non-SUMOylatable mutant of NFATc3 i.e. NFATc3-EallA using luciferase reporter assays.

Further, transcription factors often induce the expression of their E3 ubiquitin-ligases creating a negative-feedback loop. For instance, NFATc1 induces the expression of Nur77 at late stage osteoclast differentiation and Nur77, in turn, induces the expression of Cbl-b- an E3 ubiquitin-ligase of NFATc1 (Xiaoxiao Li et al. 2015). Moreover, Mdm2, an E3 ubiquitin-ligase of p53, is a target gene of p53 and once induced, targets p53 for ubiquitin-dependent proteasomal degradation (Haupt et al. 1997; Honda, Tanaka, and Yasuda 1997; Lohrum et al. 2003). Therefore, as it has been shown for Trim17 which is the target gene of NFATc3, it is possible that Trim39 itself could be a target gene of NFATc3, which in turn, creates a negative feedback loop. In our results, when we overexpressed NFATc3 alone, we did not observe any increase in Trim39 expression (data not shown), therefore, Trim39 does not seem to be a target gene of NFATc3. Finally, we cannot exclude the possibility that Trim39 might also interact with a repressor to inhibit the transcriptional activity of NFATc3.

**Apoptosis**

My group had shown that NFATc3 aggravates neuronal apoptosis (Mojsa et al. 2015b). In the present study, we have shown that compared to WT-GFP-NFATc3, the overexpression of GFP-NFATc3-EallA significantly increases neuronal apoptosis triggered by survival-factor deprivation in primary cultures of CGN. Furthermore, when we silenced endogenous Trim39, we could observe a significant increase in neuronal apoptosis compared to the control condition transduced with a non-specific control shRNA. Taken together, these results suggest that SUMO and Trim39 negatively regulate the pro-apoptotic function of NFATc3.

Interestingly, our observations point towards an anti-apoptotic function of Trim39 probably by decreasing the pro-apoptotic NFATc3 protein levels. Our results, however, do not exclude the possibility that this effect on apoptosis observed after silencing of Trim39 might be due to other factors. Few studies have highlighted the involvement of Trim39 in regulating apoptosis (S. S. Lee et al. 2009b; N.-J. Huang et al. 2012b; Lei Zhang et al. 2012b; Liguo Zhang et al. 2012b). For instance, Trim39 has been shown to induce apoptosis by elevating MOAP-1 level (Bax activator modulator of apoptosis 1) by inhibiting its ubiquitination and degradation by APC/C Cdh1. Hence, via MOAP-1 stabilization, Trim39 exerts a pro-apoptotic function. In contrast, two different studies have shown that Trim39 has an anti-apoptotic function (Lei Zhang et al. 2012b; Liguo Zhang et al. 2012b). In the first study, Trim39 has been identified as an E3 ubiquitin-ligase of p53. Interestingly, co-inhibition of Trim39 along with Nutlin-3a (inhibitor of MDM2- a well-known E3 ubiquitin-ligase of p53) has been shown to stabilize p53 and in turn, enhance apoptosis of p53+ breast cancer (MCF-7) and lung cancer cells (A549) (Liguo Zhang et al. 2012b). In the second study, Trim39 has been
shown to regulate cell cycle progression and DNA damage responses by stabilizing p21. Trim39 depletion resulted in sensitizing cells to DNA damage-induced apoptosis by decreasing the accumulation of p21 (Lei Zhang et al. 2012b). Taken together, results from other studies and our study suggest that effect of Trim39 on apoptosis might be substrate and context-dependent.

**The big picture: Schematic representation of the mechanisms regulating NFATc3**

My group had shown that Trim17 binds NFATc3 in a SUMO-dependent manner, inhibits its nuclear-translocation, and its activity (Mojsa et al. 2015b). Therefore, Trim17 should inhibit the pro-apoptotic effect of NFATc3. Moreover, Trim17 has been identified as a target gene of NFATc3, thus creating a negative feed-back loop in which Trim17 inhibits its own expression by inhibiting NFATc3. In this study, we have shown that Trim17 inhibits Trim39-mediated ubiquitination and degradation of NFATc3. This inhibitory effect of Trim17 should result in the stabilization of NFATc3 and should aggravate its pro-apoptotic effect. Moreover, this should create a positive feed-back loop by which Trim17 increases its own expression by favouring NFATc3 activity (Figure 56).

![Figure 56: Schematic representation of the effects of Trim17 on the regulation of NFATc3.](image)

We have shown that SUMOylation of NFATc3 reduces its stability and that Trim39 is a STUbL for NFATc3 which binds and ubiquitinates preferentially the SUMOylated forms of NFATc3. Further, overexpression of Trim39 reduces the transcriptional activity of NFATc3 and in contrast, silencing of Trim39 increases the expression of **Trim17** and also its pro-apoptotic effect in neurons. Taken together, our study provides an intricate mechanism of regulation of NFATc3 stability by Trim17, Trim39, and SUMO which co-ordinate the fine tuning of neuronal apoptosis (Figure 57). As NFAT are implicated in various pathologies such as auto-immune diseases, cancer, neurodegenerative diseases, further investigation will determine whether these mechanisms can be used for therapeutic purposes to improve these pathologies.
Figure 57: Schematic representation of the mechanisms by which Trim17, Trim39, and SUMO regulate the stability of NFATc3.


Implications for Human Pathobiology.”


Recruits a Novel Isoform of the Mi -


Redox Regulation of the Stability of the SUMO Protease SENP3 via Interactions with CHIP and Hsp90.” The EMBO Journal 29 (22): 3773–86. https://doi.org/10.1038/emboj.2010.245.


Résumé

Réglulation de la stabilité de NFATc3 par SUMO et les E3 ubiquitine-ligases Trim39 et Trim17

Mots-clés : facteur de transcription NFATc3, ubiquitination, SUMOylation, protéines TRIM, STUbL

Les facteurs de transcription NFAT (facteur nucléaire des cellules T activées) jouent un rôle physiologique important dans le développement et le fonctionnement de nombreux organes, notamment dans le système immunitaire et le système nerveux. Par conséquent, leur dérégulation a été impliquée dans diverses maladies humaines telles que le cancer, les maladies neurodégénératives et les maladies auto-immunes. La régulation de l'activité de NFAT par translocation nucléo-cytoplasmique a été largement étudiée. En revanche, la régulation du niveau protéique de NFAT par le système ubiquitine-protéasome est encore mal comprise. Pourtant, les protéines NFAT ont une durée de vie courte et la régulation de leur stabilité est donc essentielle pour le contrôle de leur activité.

Dans une étude précédente, mon groupe a montré que l'E3 ubiquitine-ligase Trim17 se lie à NFATc3 mais ne favorise pas son ubiquitination et tend plutôt à stabiliser la protéine. Les résultats préliminaires obtenus suggéraient que Trim39, un partenaire de Trim17, pourrait être une E3 ubiquitine-ligase pour NFATc3 et que la SUMOylation de NFATc3 modulait sa stabilité. L'objectif de ma thèse était donc de comprendre les mécanismes par lesquels Trim39, Trim17 et SUMO régulent la stabilité de NFATc3.

Au cours de ma thèse, j'ai caractérisé Trim39 comme une E3 ubiquitine-ligase de NFATc3. En effet, mes résultats indiquent que la surexpression de Trim39, mais pas de son mutant inactif, induit l'ubiquitination de NFATc3 dans les cellules. En revanche, la déplétion de Trim39 endogène diminue le niveau d'ubiquitination de NFATc3. La protéine Trim39 recombinante induit directement l'ubiquitination de NFATc3 in vitro. De plus, la surexpression de Trim39 diminue les niveaux protéiques de NFATc3 alors que la déplétion de Trim39 les augmente. J'ai également montré que Trim17 inhibe l'ubiquitination de NFATc3 induite par Trim39, à la fois dans les cellules et in vitro. Trim17 agit à la fois en réduisant l'activité E3 ubiquitine-ligase intrinsèque de Trim39 et en empêchant l'interaction entre NFATc3 et Trim39. En outre, j'ai montré qu'un mutant de NFATc3 ne pouvant être SUMOylé est moins ubiquitiné et plus stable que la forme sauvage de NFATc3, ce qui suggère que la SUMOylation de NFATc3 est importante pour son ubiquitination et sa dégradation. En outre, j'ai identifié un motif d'interaction à SUMO (SIM) dans la séquence de Trim39, par lequel Trim39 lie les polymères de SUMO2. La mutation de ce SIM dans Trim39 ou des sites consensus de SUMOylation dans NFATc3 diminue l'interaction entre Trim39 et NFATc3, et l'ubiquitination de NFATc3 induite par Trim39. Ces résultats suggèrent fortement que Trim39 reconnaît et ubiquitine préférentiellement les formes SUMOylées de NFATc3 et agit donc comme une « E3 ubiquitine-ligase guidée par SUMO » (STUbL) pour NFATc3. Enfin, nous avons mesuré l'impact de ces mécanismes sur la fonction physiologique de NFATc3. J'ai tout d'abord montré que Trim39 diminue l'activité transcriptionnelle de NFATc3. En outre, à l'aide de cultures primaires de neurones granulaires du cervelet, nous avons montré que la mutation des sites de SUMOylation de NFATc3 et la déplétion de Trim39 endogène aggravent l'apoptose neuronale, probablement en stabilisant la protéine NFATc3. En conclusion, l'ensemble de mes données indiquent que Trim39 module l'apoptose neuronale en agissant comme une STUbL pour NFATc3 et en contrôlant sa stabilité.
Abstract

Regulation of NFATc3 stability by SUMO and E3 ubiquitin-ligases Trim39 and Trim17

Keywords: NFATc3 transcription factor, ubiquitination, SUMOylation, TRIM-proteins, STUbL

NFAT (Nuclear factor of activated T cells) transcription factors play important physiological roles in the development and function of many organs, notably in the immune system and nervous system. As a consequence, their dysregulation has been implicated in various human diseases such as cancer, neurodegenerative diseases, and auto-immune diseases. The regulation of NFAT activity by calcium-dependent nuclear-cytoplasmic shuttling has been extensively studied. In contrast, the regulation of NFAT protein level by the ubiquitin-proteasome system is still poorly understood. However, NFATs are short-lived proteins and regulation of their stability is critical for controlling their activity.

In a previous study, my group has shown that the E3 ubiquitin-ligase Trim17 binds NFATc3 but does not promote its ubiquitination and rather stabilizes it. Preliminary results suggested that Trim39, a partner of Trim17, might be an E3 ubiquitin-ligase for NFATc3 and that SUMOylation of NFATc3 might modulate its stability. Therefore, the goal of my PhD was to understand the mechanisms through which Trim39, Trim17, and SUMO regulate the stability of NFATc3.

During my PhD, I have characterised Trim39 as an E3 ubiquitin-ligase of NFATc3. Indeed, my results indicate that overexpression of Trim39, but not its inactive mutant, induces the ubiquitination of NFATc3 in cells. In contrast, silencing of endogenous Trim39 decreases the ubiquitination level of NFATc3. Recombinant Trim39 directly induces the ubiquitination of NFATc3 in vitro. Moreover, overexpression of Trim39 decreases the protein levels of NFATc3 whereas silencing of Trim39 increases it. I have also shown that Trim17, which can bind Trim39, inhibits Trim39-mediated ubiquitination of NFATc3, both in cells and in vitro. Trim17 acts by both reducing the intrinsic E3 ubiquitin-ligase activity of Trim39 and by preventing the interaction between NFATc3 and Trim39. Furthermore, I found that a SUMOylation-deficient mutant of NFATc3 is less ubiquitinated and more stable than the wild type NFATc3, suggesting that SUMOylation of NFATc3 is important for its ubiquitination and degradation. Importantly, I identified one SUMO interacting motif (SIM) in the sequence of Trim39 through which Trim39 binds SUMO2 polymers via one of these SIMs. Mutation of this SIM in Trim39 or SUMOylation consensus sites in NFATc3 decreased the interaction between Trim39 and NFATc3, and the ubiquitination of NFATc3 mediated by Trim39. These results strongly suggest that Trim39 binds and ubiquinates preferentially the SUMOylated forms of NFATc3 and therefore acts as a SUMO-targeted E3 ubiquitin-ligase (STUbL) for NFATc3. Finally, we have measured the impact of these mechanisms on the physiological function of NFATc3. I first found that Trim39 decreases the transcriptional activity of NFATc3. Furthermore, using primary cultures of cerebellar granule neurons as a model, we have shown that the mutation of the SUMOylation sites of NFATc3 and silencing of endogenous Trim39 enhances neuronal apoptosis, probably by stabilizing the NFATc3 protein. Taken together, these data indicate that Trim39 modulates neuronal apoptosis by acting as a STUbL for NFATc3 and by controlling its stability.