The impact of sumoylation on the interaction between Nfi-SecPH domain and its interaction partners: VCP/p97 and LIMK2

Marjanović, Lovro

Master's thesis / Diplomski rad

2023

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Zagreb, Faculty of Food Technology and Biotechnology / Sveučilište u Zagrebu, Prehrambeno-biotehnološki fakultet**

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:159:134828

Université d'Orléans - Université de Zagreb

UFR Sciences et Techniques - Faculté de Nutrition et Biotechnologie - Faculté des Sciences

MASTER SCIENCES DU VIVANT

Spécialité : Techniques Bio-Industrielles

INTERNSHIP REPORT

THE IMPACT OF SUMOYLATION ON THE INTERACTION BETWEEN Nf1-SecPH DOMAIN AND ITS BINDING PARTNERS: VCP/p97 AND LIMK2

By

Lovro Marjanović

(February 2023 – June 2023)

Internship institution and address: **Centre de Biophysique Moléculaire - UPR 4301, Rue Charles Sadron CS 80054, 45071 Orleans**

Team: **Cell Signaling and Neurofibromatosis**

Mentor/supervisor: **Dr. Sc. Christine Mosrin-Huaman, Researcher**

Zagreb, June 2023

ACKNOWLEDGMENTS

I would like to express my heartfelt gratitude to Dr. Sc. Christine Mosrin-Huaman, my mentor, for her invaluable guidance, teaching, and patience during my internship. With the knowledge she has passed to me, I am now one step closer to realizing my dream of becoming a scientist.

I extend my sincerest thanks to Dr. Sc. Hélène Bénédetti and Dr. Sc. Beatrice Vallée for accepting me into their team and dedicating their time to guide and advise me at every step of the journey.

I am immensely grateful to all the members of the 'Cell Signaling and Neurofibromatosis' team: Amandine Serrano, Elodie Villalonga, Fabienne Godin, Michel Doudeau, and Thierry Normand, for their tremendous help and for making me feel welcomed as part of the team.

Furthermore, I would like to express my appreciation to our professors: Josef Hamacek, Igor Stuparević, Višnja Besendorfer, Vladimir Mrša, and Chantal Pichon, for organizing and conducting this incredible study and for always being available to assist when needed.

Lastly, I want to thank my family and friends who have been a constant source of support throughout my life and have played a significant role in shaping the person I am today.

To all of you, thank you!

TABLE OF CONTENT

‘CELL SIGNALING AND NEUROFIBROMATOSIS’ TEAM

The research was conducted under the mentorship of Dr. Sc. Christine Mosrin-Huaman as a part of the ‘Cell Signaling and Neurofobromatosis’ thematic group led by Dr. Sc. Hélène Bénédetti and Dr. Sc. Beatrice Vallée. The thematic group is part of the CBM (Centre for Molecular Biophysics or Centre de Biophysique Moléculaire) laboratory located in Orleans, France. CBM is part of CNRS - French National Centre for Scientific Research (Centre National de la Recherche Scientifique).

The team is primarily interested in neurofibromatosis type 1 (NF1), a disease with various manifestations. One significant aspect for further investigation is the heightened risk of malignancies and neurological disorders in individuals with NF1. Currently, the team is studying cell signaling and exploring novel interaction partners of a protein called neurofibromin (Nf1), which is encoded by the *NF1* tumor-suppressor gene and mutated in NF1 patients. The focus is specifically on the recently discovered interaction partners of Nf1, namely LINGO-1, LARP6, and LIMK2. Additionally, their focus is also set on uncovering posttranslational modifications like sumoylation and ubiquitination of neurofibromin and understanding their role in both healthy and diseased conditions.

LIST OF ABBREVIATION

NF	Neurofibromatosis
NF1	Neurofobromatosis type 1
NF2	Neurofibromatosis type 2
Nf1	Neurofibromin protein
cNFs	Cutaneous Neurofibromas
pNFs	Plexiform Neurofibromas
MPNSTs	Malignant Peripheral Nerve Sheath Tumors
CSRD	Cysteine-Serine-rich domain
GRD	GAP-related domain
GAP	GTPase Activating Protein
TBD	Tubulin-Binding Domain
PH	Pleckstrin Homology like Domain
CTD	C-terminal domain
cAMP	Cyclic Adenosine Monophosphate
RAS	proto-oncogene protein p21
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
RAF	Rapidly Accelerated Fibrosarcoma
MEK	Mitogen-Activated Protein Kinase
ERK	Extracellular Signal-Regulated Kinase
PI3K	Phosphoinositide 3-kinase
AKT	AKT serine/threonine Kinase
mTOR	Mammalian Target Of Rapamycin
RHO	small GTPase Rho
ROCK	RHO-associated, Coiled-coil-forming protein kinase
LIMK2	LIM domain kinase 2
RAC1/PAK1	P21 Activated Kinase
LIMK1	LIM domain kinase 1
ADF	Actin Depolymerization Factor
VCP	Valosin-Containing Protein
AAA	ATPase Associated with a Variety of Cellular Activities
IBMPFD	Inclusion Body Myopathy with Paget Disease of the Bone and Frontotemporal Dementia
PTM	Post-Translational Modifications
SUMO	Small Ubiquitin-like modifier
SIM	SUMO-interacting motifs
SENP	sentrin-specific protease
SAE1/SAE2	SUMO-activating enzyme subunits 1 and 2
PPIs	protein-protein interactions
PML-NB	ProMyelocytic Leukemia Nuclear Bodies
LRD	Leucine-rich domain
HLR	Heat-Like Repeat
HEK293	Human Embryonic Kidney 293 cell line
Ubc9	Ubiquitin Conjugating enzyme 9
Mdh1	Malate Dehydrogenase 1
5-HT6	Serotonin 5-Hydroxytryptamine 6 Receptor

1. INTRODUCTION

1.1. NEUROFIBROMATOSIS

Neurofibromatosis (NF) are disorders that predominantly impact tissues originating from the neuronal crest, including skin, nerves, and bones. A shared characteristic among these disorders is the development of tumors on any part of the nerve tissue. These disorders include neurofibromatosis type 1 (NF1), also known as von Recklinghausen disease, neurofibromatosis type 2 (NF2), and schwannomatosis (Shen et al. 1996). The most common of these three diseases is neurofibromatosis type 1 (NF1), affecting 1 in 3000 individuals worldwide. NF1 is an autosomal dominant disease caused by inherited or de novo germline mutations in the *NF1* tumor-suppressor gene. The *NF1* gene is located at chromosome 17. It is a very large gene spanning 350 kb of coding DNA which contains 60 exons that encode for neurofibromin protein (Nf1). Nf1 belongs to a family of proteins that serve as negative regulators of the Ras oncogene (Rasmussen & Friedman, 2000). It is the most frequently diagnosed tumor predisposition disorder in humans (Cimino & Gutmann 2018). Characteristic features of the disease include pigmentary lesions and diverse types of tumors affecting the peripheral nervous system, such as cutaneous neurofibromas (cNFs), plexiform neurofibromas (pNFs), and malignant peripheral nerve sheath tumors (MPNSTs). Nevertheless, some individuals may also experience additional symptoms, such as skeletal abnormalities, brain tumors, learning disabilities, attention deficit, as well as social and behavioral issues (Bergoug et al. 2020).

To date, more than 3000 different pathogenic alterations have been documented in the *NF1* gene. They are dispersed throughout the entire gene and are without any particular hot spot. Large deletions in the gene are observed in 5 to 10 percent of NF1 patients, often associated with a more severe phenotype. For patients with intragenic missense *NF1* mutations, no clear correlations between specific mutations and clinical manifestations have been established thus far.

1.2. NEUROFIBROMIN (Nf1) PROTEIN

Neurofibromin (Nf1) is a large multi-domain protein consisting of 2818 amino acids. It contains various domains, including a cysteine-serine-rich domain (CSRD) at the N-terminus, a GAP-related domain (GRD) with a tubulin-binding domain (TBD) in the central region, a phospholipid and protein interaction domain called SecPH, and a C-terminal domain (CTD) at

the end. The expression and functions of Nf1 are regulated at multiple levels, including transcriptional control, RNA processing, mRNA transport, protein targeting, and protein degradation (Peltonen et al. 2017). The most well-studied function of Nf1 is its Ras-GAP (GTPase activating protein) activity performed by the GRD domain. This activity involves stimulating the intrinsic GTPase activity of Ras. However, this is just one aspect of its extensive protein structure. Recent investigations into Nf1's binding partners have begun to uncover its connections to various signaling pathways and its involvement in regulating cytoskeleton dynamics, cAMP levels, dendritic spine density, neurite outgrowth, and microtubule-dependent transport (Figure 1).

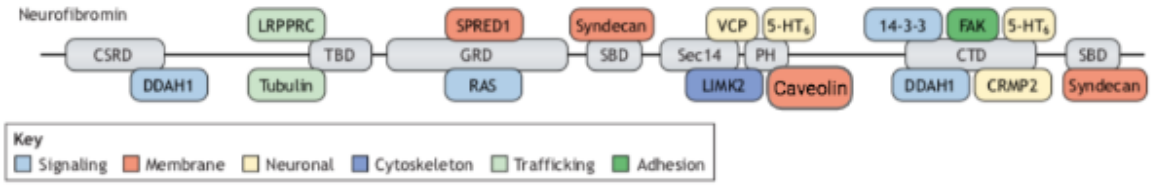

Figure 1. The schematical representation of Nf1 domains and its various interacting partners connect Nf1 to different signaling pathways. Source: Anastasaki et al., 2022

1.2.1. GRD DOMAIN AND RAS-GAP ACTIVITY

The main role of Nf1 is to act as a tumor-suppressor protein by carrying out Ras-GAP activity. This activity involves increasing the GTPase activity of Ras, leading to the breakdown of active Ras-GTP into inactive Ras-GDP. As a result, the Ras/Raf/MEK/ERK and Ras/PI3K/Akt/mTOR signaling pathways, which are responsible for cell proliferation, growth, and survival, are downregulated (Figure 2) (Bergoug et al., 2020). Nf1's Ras-GAP function is crucial in regulating the cell cycle and tissue growth, especially in cancer studies, where mutations in the Ras pathways drive tumor formation. Loss of *NF1* heterozygosity, resulting in the loss of Nf1 expression and Ras-GAP activity, is a key step for both benign and malignant tumor development. This loss of function leads to the accumulation of Ras-GTP, excessive signaling through the Ras pathways, and uncontrolled cell proliferation, growth, and resistance to apoptosis, ultimately resulting in tumor development (Anastasaki et al. 2022).

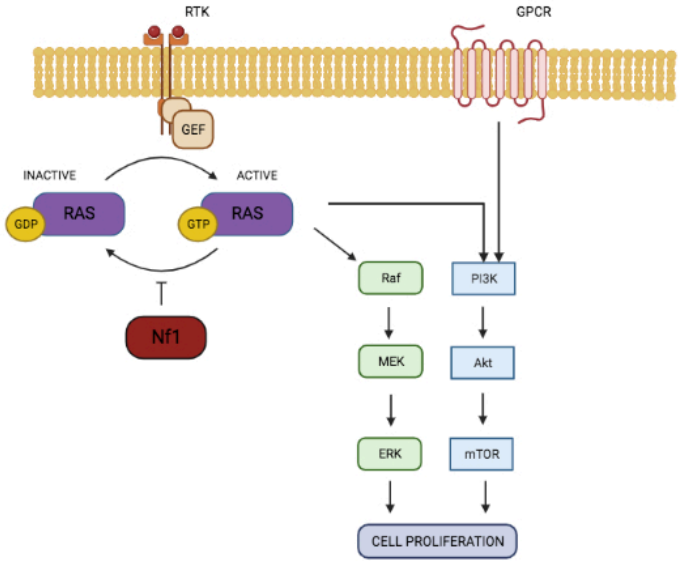

Figure 2. The figure illustrates the modulation of different signaling pathways by Ras-GTP. Nf1, through its GRD domain, functions as a Ras-GAP, thereby suppressing cell proliferation and survival.

1.2.2. SecPH DOMAIN

The SecPH domain in Nf1 consists of two modules: a Sec14-like domain, similar to the lipid-binding domain of yeast phosphatidylinositol transfer protein Sec14p, and a pleckstrin homology like domain (PH) for protein-protein interactions (PPIs). The 3D structure of SecPH was resolved in 2006, revealing a partially helical linker peptide connecting the two modules, called β-protrusion (D'Angelo et al. 2006). While the exact function of SecPH is not fully understood, it has been shown to bind phospholipids and proteins. These protein interactions link Nf1 to various signaling pathways. The role of phospholipid interactions remains unclear, but it may enable Nf1 to interact with membranes and be in close proximity to Ras. Furthermore, due to its proximity to the GRD domain, the SecPH domain may contribute to fine-tuning Nf1's Ras-GAP activity (Bergoug et al. 2020).

1.2.3. LIM KINASES AND CONTROL OF ACTIN CYTOSKELETON ORGANIZATION

In addition to its Ras-GAP activity, Nf1 also performs a critical role in the regulation of cytoskeletal organization. More specifically, Nf1 regulates actin filaments' dynamic reorganization and turnover by negatively regulating two parallel pathways: the Rho/ROCK/LIMK2/Cofilin pathway and the Rac1/PAK1/LIMK1/Cofilin pathway. Both LIM kinases (LIMK1 and LIMK2) share the same domain organization, consisting of two amino-

terminal LIM domains, an adjacent PDZ domain, a serine/proline (SP)-rich domain, and a carboxyl-terminal kinase (KIN) domain (Okano et al., 1995). Precise regulation of actin polymerization/depolymerization is crucial for many essential biological processes, such as cell motility and morphology. Cofilin, a member of the ADF family (actin depolymerization factor), severs aging actin filaments to promote depolymerization. However, LIM kinases inhibit this process by phosphorylating cofilin, thereby stabilizing actin filaments, promoting stress fiber formation, and increasing focal adhesion (Scott & Olson, 2007). In 2005, Ozawa et al. demonstrated that Nf1 negatively regulates the Rho/ROCK/LIMK2/cofilin pathway and that siRNA targeted against the *NF1* gene led to the prolonged phosphorylation and subsequent inactivation of cofilin by LIMK2, thereby promoting the formation of focal adhesions and stable actin stress fibers. Vallee et al. (2012) uncovered the detailed molecular mechanism of Nf1 on the Rho/ROCK/LIMK2/Cofilin pathway. They showed that the SecPH domain of Nf1 interacts with LIMK2 thereby preventing LIMK2 activation through its upstream regulator, ROCK, and the further phosphorylation of cofilin. In the absence of phosphorylation, cofilin retains its active state and is capable of depolymerizing actin (Figure 3). This, in turn, reduces the accumulation of actin stress fibers induced by LIMK2 (Vallée et al., 2012). In addition, Nf1 also acts as an inhibitor of the parallel signaling pathway Rac1/PAK1/LIMK1/cofilin. The pre-GRD domain of Nf1 ($Nf1_{1-1163}$) is involved in this process but the detailed molecular mechanism involved is unknown (Starinsky-Elbaz et al. 2009).

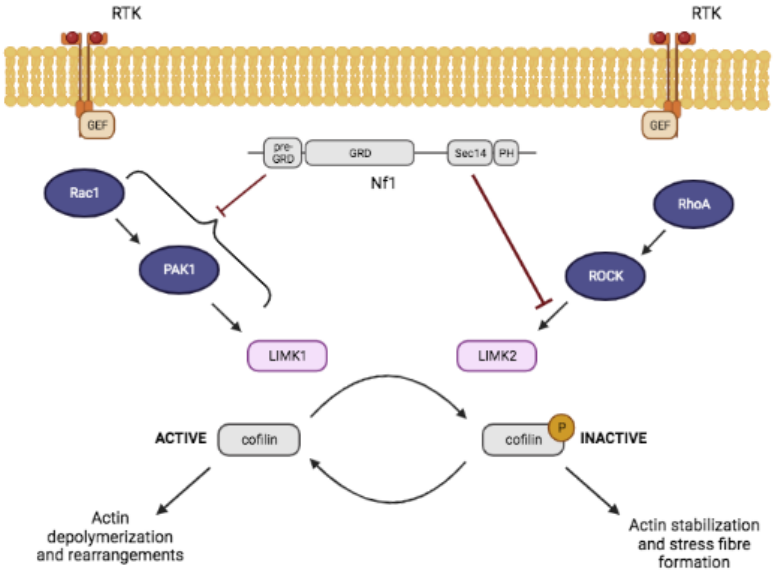

Figure 3. Regulation of Rac1/PAK1/LIMK1/Cofilin and Rho/ROCK/LIMK2/Cofilin pathway by Nf1.

1.2.4. VALOSIN-CONTAINING PROTEIN (VCP) AND REGULATION OF SPINE DENSITY

Valosin-containing protein (VCP), also known as p97, is a versatile AAA (ATPase associated with a variety of cellular activities) protein that plays a role in numerous cellular processes, including DNA replication, mitosis, protein degradation, endocytosis, membrane fusion, and organelle biogenesis (Halawani & Latterich 2006). The diverse functions of VCP stem from its ability to interact with multiple binding partners. Currently, over 50 proteins have been identified as direct or indirect associates of VCP through ubiquitin adaptors. However, the specific functions of many of these interactions remain elusive (Dreveny et al. 2004). Mutations in the *VCP* gene cause IBMPFD (Inclusion Body Myopathy with Paget disease of the bone and Frontotemporal Dementia), a disorder characterized by muscle weakness and dementia. VCP plays a crucial role in maintaining cellular balance, but mutations disrupt this process, making affected cells more vulnerable to degradation (Watts et al. 2004). In a study by Wang et al. in 2011, Nf1 was identified as a novel interacting protein of VCP. When VCP was depleted in rat hippocampal neurons, it resulted in reduced dendritic spine density, similar to the effects seen with Nf1 depletion in mice. This suggests that VCP and Nf1 may function together in a pathway related to synaptogenesis. However, the exact mechanism of their regulation in synaptogenesis remains unclear.

1.3. POST-TRANSLATIONAL MODIFICATIONS OF NEUROFIBROMIN

The Nf1 protein is involved in essential cellular processes and interacts with various partners and thus undergoes rigorous regulation at the level of expression and by post-translational modifications (PTM). These PTMs include phosphorylation, which influences its interactions with specific partners (Feng et al. 2004), ubiquitination, leading to proteasomal degradation (Cichowski et al. 2003), and a newly discovered PTM called SUMOylation (currently being studied by the Cell Signaling and Neurofibromatosis team).

1.3.1. SUMOYLATION

SUMOylation is a type of post-translational modification where small Ubiquitin-like modifiers (SUMOs) are covalently attached to specific lysine residues in proteins. SUMO is a family of highly conserved small-molecule proteins that are prevalent in eukaryotes. Currently, five SUMO proteins (SUMO1-5) are identified in eukaryotes, with a molecular weight of approximately 11 kDa (Zhong et al. 2023). SUMO2 and SUMO3 share such a high sequence

similarity of 97% that antibodies cannot distinguish them. Therefore, they are collectively referred to as SUMO2/3. However, SUMO2/3 and SUMO1 exhibit only 46% sequence similarity (Chang & Yeh, 2020; Yuan et al., 2018). SUMO2 and SUMO3 can form conjugated chains by utilizing a single conserved acceptor lysine, thereby leading to polySUMOylation. Furthermore, SUMO2 possesses multiple lysines on its surface that can be utilized for these chain formations. On the other hand, an extensive investigation into SUMO targets under normal conditions and proteostasis stress has revealed that SUMO1 is also capable of forming linear monomeric chains, but predominantly conjugates as a single molecule to a targeted protein (Hendriks et al. 2017). SUMO1, SUMO2, and SUMO3 are widely expressed in all cells and organs, while SUMO4 displays specific expression in certain organs such as the kidney, lymph nodes, and spleen (Han et al. 2018). Additionally, SUMO5 is primarily expressed in the lung and spleen (Liang et al. 2016).

The SUMOylation pathway is similar to ubiquitination but utilizes distinct enzymes. SUMO proteins undergo processing by a family of proteases called sentrin-specific proteases (SENPs), leading to their maturation and exposure of the C-terminal di-glycine (-GG) motif necessary for conjugation (Mukhopadhyay & Dasso 2007). Once matured, the SUMO proteins are activated, forming an adenylate adduct with the heterodimeric E1 and E2 activating enzymes (SAE1/SAE2) through ATP-dependent reactions. SAE1/SAE2 catalyzes the formation of a high-energy thioester bond between the C-terminal end of the SUMO protein and a cysteine residue within its active site. The activated SUMO is then transferred to the ubiquitin-conjugation enzyme Ubc9, either independently or in conjunction with SUMO E3 ligases. Ubc9, working with SUMO E3 ligases or alone, facilitates the conjugation of activated SUMO to a targeted lysine residue, typically found within a consensus sequence ψ-K-X-D/E (where ψ represents a large hydrophobic amino acid, X represents any amino acid, K represents lysine, and D/E represents aspartic acid or glutamic acid) or an inverted consensus sequence. Importantly, the SUMOylation process is reversible, providing dynamic regulation. DeSUMOylation, the removal of SUMO modifications, is carried out by SENPs, ensuring fine-tuning of SUMOylation levels and allowing for precise control of protein functions and interactions (Figure 4). This intricate interplay between the SUMOylation and deSUMOylation steps contributes to the complex regulatory landscape of cellular processes (K et al. 2021).

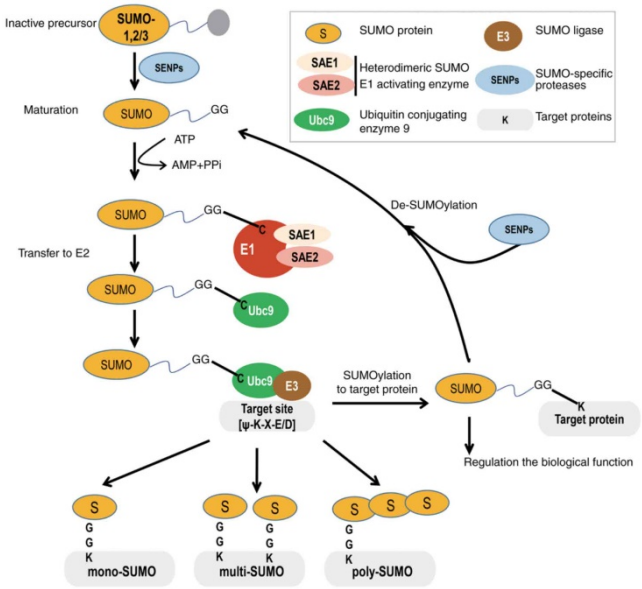

Figure 4. Process of SUMOylation in mammalians. Source: Wang et al., 2021

In addition to the covalent modifications of SUMO proteins, non-covalent interactions can also occur between SUMOs and their target proteins or interactors through SUMO-interacting motifs (SIMs). SIMs are found in various proteins and come in different types, playing a central role in recognizing and binding to SUMO. Typically, SIMs consist of a hydrophobic core with a sequence pattern of (V/I) – X – (V/I) – (V/I) (V- Valin; I – Isoleucine), flanked by a negatively charged amino acid. The interaction between SUMO and SIM has been shown to take place between the hydrophobic core of the SIM and a surface region on the SUMO protein. Furthermore, the adjacent acidic residues in the SIM are believed to contribute to the affinity of the interaction (K et al. 2021). The dynamic attachment of SUMO to target proteins has been implicated in diverse cellular events. It participates in protein-protein interactions, precise intracellular localization of proteins, plays a role in DNA repair processes, is involved in nucleocytoplasmic transport, and additionally, impacts apoptosis, influencing cell survival and programmed cell death mechanisms (Zhong et al. 2023).

1.4. PREVIOUS STUDIES

1.4.1. ENDOGENOUS Nf1 IS SUMOYLATED BY SUMO2

In 2012, Godin et al. provided the first evidence of Nf1 SUMOylation in their research team. They observed partial co-localization of Nf1 with PML-NB (ProMyelocytic Leukemia

Nuclear Bodies) which are known to contain SUMOylation machinery. This finding suggested that PML bodies could serve as "hot spots" for the SUMOylation of Nf1. To confirm Nf1 as a genuine substrate for SUMOylation, an experiment was conducted using HEK293 cells that naturally express Nf1. The cells were co-transfected with two plasmids, one encoding for SUMO conjugating enzyme Ubc9 and the other Flag-tagged SUMO2. This way, the SUMO2-conjugation to the targeted protein is enhanced inside the cells. Immunoprecipitation method using anti-Flag was utilized to capture SUMOylated proteins and SUMO-interacting proteins. Subsequently, a second immunoprecipitation was performed using anti-Nf1 antibodies to enrich Nf1-derived proteins. Western blot analysis using both anti-Flag and anti-Nf1 antibodies confirmed the presence of a band at approximately 300 kDa, corresponding to the molecular mass of Nf1. This observation provided conclusive evidence that Nf1 is genuinely SUMOylated by SUMO2 (Figure 5).

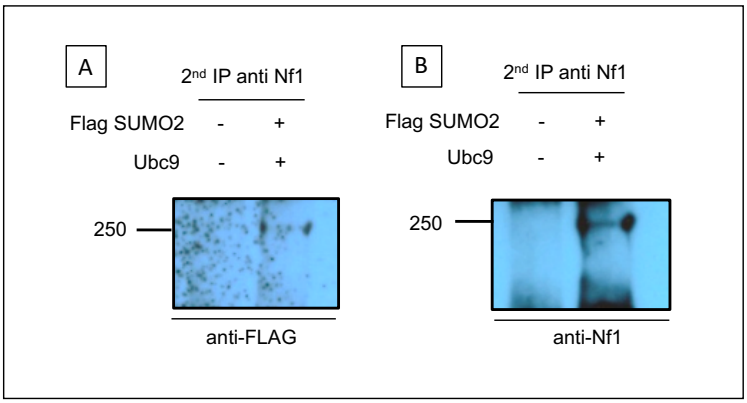

Figure 5. Endogenous Nf1 is SUMOylated by SUMO2 as shown by Western blot analysis of the eluates of the second immunoprecipitation with anti-Nf1 antibodies. A. Western blot analysis of the eluate using an anti-Flag antibody in cells transfected with Flag-tagged SUMO2 and Ubc9. A band was detected, at approximately 300 kDa corresponding to the size of Nf1. **B.** As well in the first picture, in cells transfected with Flag-tagged SUMO2 and Ubc9, a band was detected with an anti-Nf1 antibody, proving that Nf1 is indeed SUMOylated by SUMO2.

1.4.2. K1731 IS IMPORTANT FOR SecPH SUMOYLATION

The Nf1 sequence was analyzed using SUMOPLOT and JASSA, two SUMOylation prediction software programs, to find potential SUMOylation sites (Figure 6A). The SecPH domain of Nf1 had the most predicted SUMOylation consensus sites and was selected for further investigation. It was chosen because of its known 3D structure, proximity to the GRD domain involved in Ras-GAP activity, and association of pleckstrin homology domain (PH) as

an enriched SUMOylation substrate. To confirm SecPH SUMOylation, 6xHis-pull-down experiments were performed using cobalt beads after transfecting HEK293 cells with a plasmid containing Flag-tagged SecPH and two additional plasmids for overexpressing Ubc9-V5-tagged and 6xHis-tagged SUMO2. Co-transfecting cells with Ubc9 and SUMO2 plasmids enhances SUMO-conjugation, resulting in more SUMO2 on targeted proteins. Four bands were observed with anti-Flag antibodies against Flag-SecPH (Figure 6B). Bands 1, 2, and 3 migrated at around 50 kDa, roughly corresponding to the mass of SecPH (37 kDa) plus one SUMO molecule (11 kDa), while band 4 had a slightly higher molecular weight. Next, specific lysine residues within the predicted SUMOylation sites were mutated to arginine, a similar residue that does not undergo SUMOylation (Figure 6C). The strongest band, band 2, disappeared in the SecPH K1731R mutant, indicating its importance in SecPH SUMOylation.

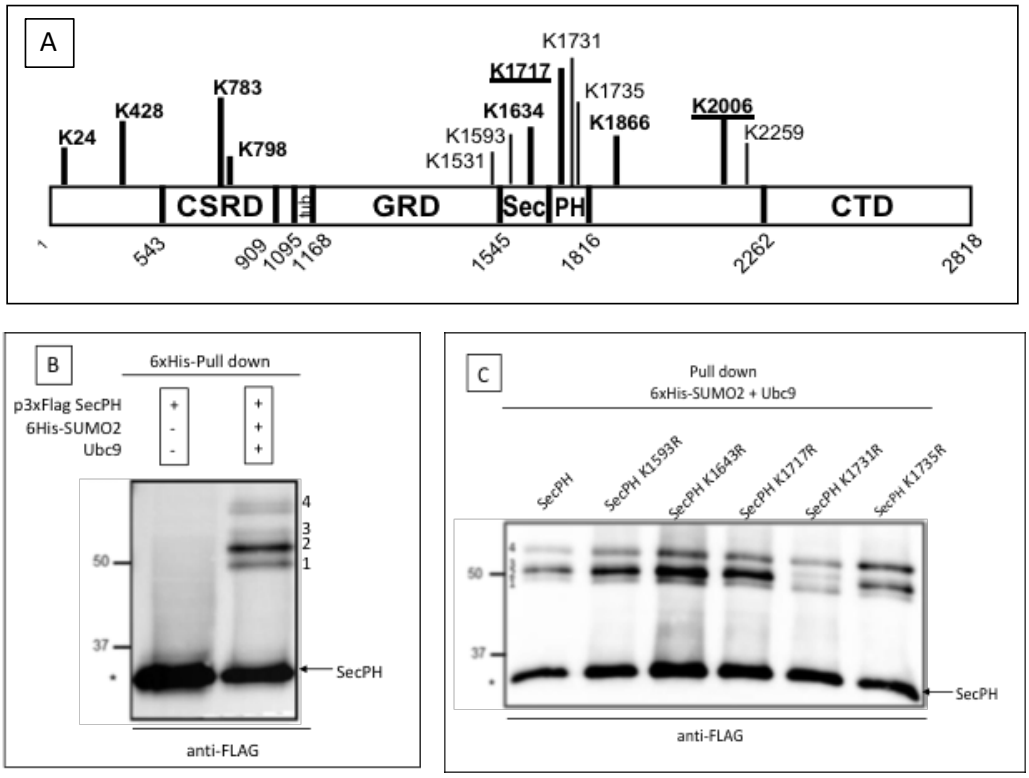

Figure 6. K1731 is a major SUMOylation site on the SecPH. A. Localization of putative SUMO acceptor lysines in Nf1 predicted by JASSA and SUMOPLOT software. **B.** Western blot results of the 6xHis-pull-down eluates of SecPH WT. The results show the specific SUMOylation profile of the SecPH domain. **C.** Western blot results of the pull-down eluates of SecPH derivates in which one out of five lysines was individually substituted with arginine (K1593R, K1634R, K1717R, K1731R, and K1735R). * in B and C represents unSUMOylated SecPH bound non-specifically to beads

1.4.3. PATIENT MISSENSE MUTATIONS AFFECT THE SUMOYLATION PROFILE OF THE SecPH

Mutations in the *NF1* gene are linked to neurofibromatosis type I, but their specific impact on the phenotype is largely unknown. Additionally, one of these mutations is K1731R and is of particular interest, suggesting a potential involvement of SUMOylation in disease development, although its clinical significance remains unclear. Further investigation into the structural determinants of K1731 SUMOylation revealed that the surrounding SUMOylation consensus site is not relevant for its SUMOylation. Instead, the SecPH β-protrusion, which is structurally close to K1731, was found to play a role in its SUMOylation. Pathogenic mutations in this β-protrusion, such as R1784P, R1784Q, and K1750E, abolished K1731 SUMOylation, providing additional support for the involvement of SUMOylation in disease development (Figure 7A). The team also examined the impact of various pathogenic mutations within the SecPH domain. Mutations like S1578F, C1661R, L1602R, D1623G, Δ1719-1736, A1764, T1787M, and R1809C were introduced. Some of these mutations affected SecPH SUMOylation and exhibited distinct SUMOylation profiles compared to both wild-type and K1731R mutant. Specifically, five mutants (S1578F, L1602R, D1623G, C1661R, and Δ1719-1736) showed an atypical SUMOylation profile (Figure 7B). All of these mutants exhibit a lower expression level of the SecPH, suggesting that these mutations cause the unfolding of the protein leading to its degradation.

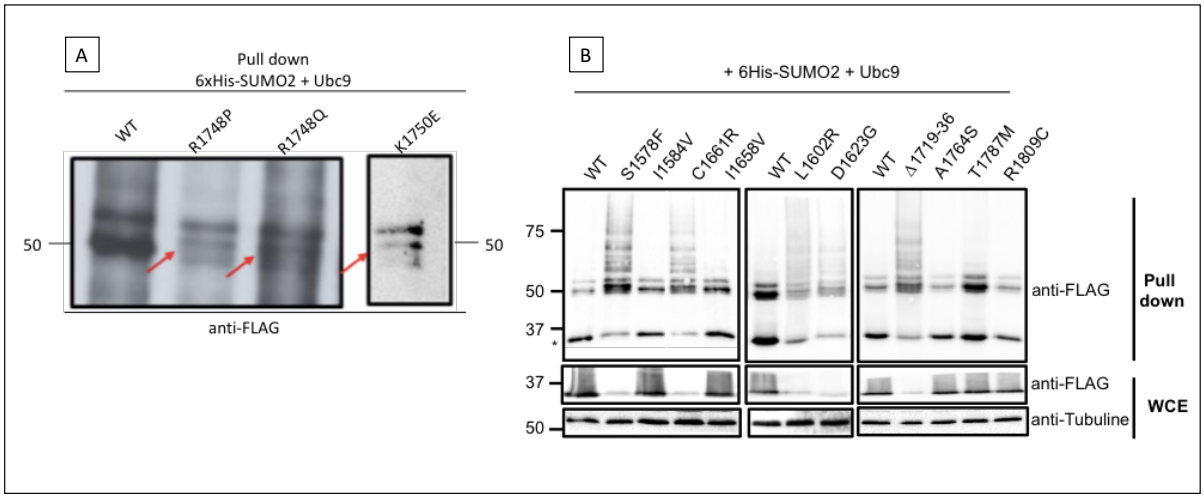

Figure 7. Patient missense mutations and deletions affect the SUMOylation profile of the SecPH. A. SUMOylation profiles of SecPH carrying patient mutations in the β-protrusion. The red arrows show the loss of K1731 SUMOylation. **B.** SUMOylation profiles of additional patient mutations.

1.4.4. SUMOYLATION DOES NOT AFFECT RAS-GAP ACTIVITY

Due to its proximity to the GRD domain, SecPH has been proposed to allosterically regulate the Ras-GAP activity of the GRD domain in Nf1. Recently, the team successfully cloned the entire human *NF1* gene into an expression vector, allowing them to investigate the complete Nf1 protein in their experiments rather than focusing solely on specific domains. To assess the impact of SUMOylation on the Ras-GAP activity of Nf1, the team examined the Ras-GAP activity of the K1731R mutant. They measured the P-ERK/ERK ratio through western blotting and quantified the Nf1-mediated regulation of Ras-GTP levels using the Ras G-LISA® kit. Double *NF1* knockdown HeLa cells were transfected with a plasmid carrying the wild-type *NF1* gene and *NF1* gene carrying K1731R mutation independently to test this. In the absence of the *NF1* gene, the Ras/Raf/MEK/ERK pathway is overactivated, resulting in elevated levels of P-ERK and Ras-GTP. Interestingly, the Nf1 K1731R mutant exhibited a similar Ras-GAP activity to wild-type Nf1 (Figure 8A). The findings indicate that the SUMOylation of K1731 does not impact the Ras-GAP activity of Nf1. This suggests that other functions of Nf1, such as its interactions with various SecPH partners, could be affected by SecPH SUMOylation. This discovery highlights a novel aspect of Nf1's functionality that is distinct from the extensively studied Ras activity.

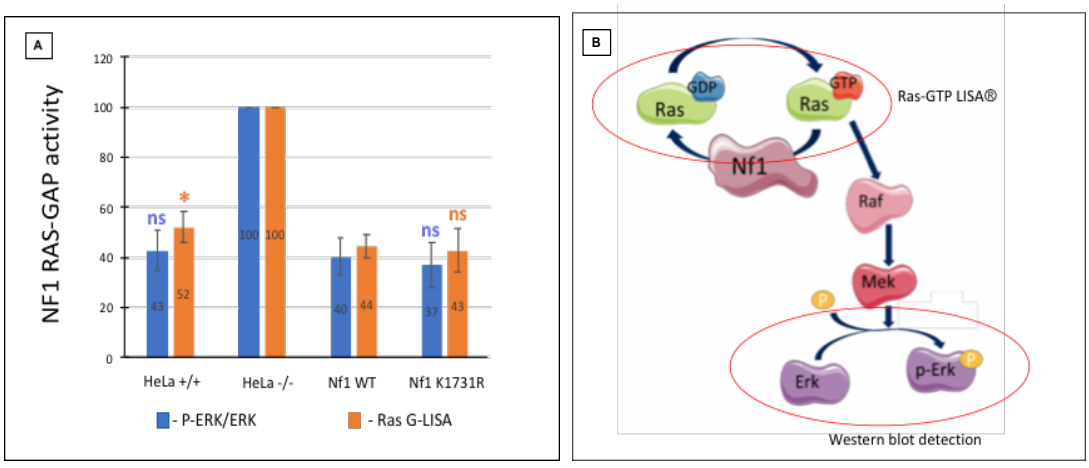

Figure 8. Analysis of Ras-GAP activity of K1731R mutant. A. Levels of P-ERK/ERK and Ras-GTP in WT and double *NF1* knockdown HeLa mutant and in double knockdown HeLa cells after transfecting them with full-length WT *NF1* and *NF1* carrying K1731R mutation. **B.** Graphic depiction of what was measured for determining the Ras-GAP activity.

1.5. NEW OBJECTIVES

The impact of K1731 SUMOylation on the function of Nf1 remains uncertain, as it does not affect its Ras-GAP activity. However, considering the hypothesis regarding SecPH's interactions with binding partners and the location of K1731 within this domain, it is speculated that its SUMOylation might modulate these interactions, either enhancing or reducing them. To explore this hypothesis, my internship focused on examining interactions with two extensively characterized SecPH binding partners, VCP and LIMK2. This groundbreaking study has the potential to provide valuable insights into the broader functional implications of SUMOylation in shaping Nf1's function.

2. MATERIALS AND METHODS

---

1. PLASMID PREPARATION

All the plasmids used in this study are listed in Table 1. Firstly, 10-beta competent *E. coli* cells (New England Biolabs, C3019H) were individually transformed with each of the plasmids using the High-Efficiency Transformation Protocol provided by New England Biolabs. The transformed cells were then plated on Luria-Bertani (LB) agar (LB: 5 g/L NaCl, 16 g/L tryptone, 10 g/L yeast extract, 15 g/L agar) with ampicillin at a final concentration of 100 µg/mL. After 24 hours at 37°C, single colonies were selected, inoculated into 3 mL of LB media with ampicillin, and allowed to grow for 8 hours at 37°C. Subsequently, they were transferred to Erlenmeyer flasks containing 200 mL of LB media with ampicillin and grown overnight at 37°C. The plasmids were purified using the *NucleoBond Xtra Midi kit* (Macherey-Nagel, 740410.50). Once purified, the plasmids were sequenced at EurofinsGenomics with primers to determine if the plasmid sequences were adequate.

2. CELL GROWTH AND TRANSFECTION

The HEK293 cell line, derived from human embryonic kidney cells, was used as an expression system in this study. The cells were cultured in 10-cm plates in low-glucose Dublecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, D6046) supplemented with 10% fetal bovine serum (FBS-Gibco) at 37°C under 5% CO_2 conditions. 24 hours before transfection, the cells were seeded onto 10 cm plates coated with a collagen layer. Transfection was carried out using calcium-phosphate co-precipitation ($CaCl_2$) with BES (Jordan & Wurm,

2004). The plasmid DNA (pDNA) used for transfection was prepared in TE (Tris -EDTA, pH 8.0) buffer and diluted to a final concentration of 20 µg. Subsequently, BES was added to the pDNA solution, which was then incubated at room temperature for 30 minutes. After the incubation period, the mixture was introduced to the cells. Following 24 hours of transfection, the media was replaced, and after an additional 48 hours, the cells were harvested for subsequent analysis. Three different conditions were used to investigate the effect of SUMOylation: endogenous, where SUMOylation naturally occurs within the cells, enhanced, where SUMOylation is stimulated, and in conditions where the SUMOylation pathway is inhibited. In the endogenous SUMOylation experiments, the cells were co-transfected with two plasmids: 20 µg of p3xFlag-Myc-CMV-24-SecPH or p3xFlag-Myc-CMV-24-SecPH K1731R, which carries DNA sequences of the wild-type SecPH domain (SecPH WT) or SecPH carrying a substitution mutation K1731R, and 20 µg of pcDNA3 2xHA LIMK2a, which contains the DNA sequence of LIMK2a (the full-length LIMK2 isoform) tagged with two hemagglutinin tags (HA). Alternatively, the cells were transfected with 20 µg of pEGFP-N1 VCP-EGFP-6xHis-HA (Table 1.), which contains the DNA sequence for VCP tagged with EGFP, a 6-histidine tag, and a hemagglutinin tag (HA). To enhance SUMOylation, the cells were transfected with the aforementioned plasmids and additionally with 10 µg of pcDNA3 containing DNA sequence of Ubc9 and 10 µg of pcDNA3 containing SUMO2. Overexpression of Ubc9 and SUMO2 ensured sufficiently high levels of SUMOylation and predominantly facilitated SUMOylation by the SUMO2 protein, minimizing the influence of other SUMO proteins. To suppress the SUMOylation pathway, a commercially available inhibitor, ML792, was used. This inhibitor specifically targets the heterodimeric E1 and E2 (SAE1/SAE2) activating enzymes. It was introduced into the media 18 hours before cell harvest, in a final concentration of 400 nM.

3. FLAG IMMUNOPRECIPITATION

Flag immunoprecipitation was used to capture the proteins interacting with Flag-tagged SecPH. To accomplish this, Flag beads (ANTI-FLAG M2 Affinity Gel, Sigma-Aldrich, A2220) were used as the capturing agent. The first step involved the preparation of the beads for immunoprecipitation by subjecting them to a series of washes. The beads were washed three times with 1% TENET (30 mM Tris-HCl pH 7.5, 120 mM NaCl, 5 mM EDTA pH 8.0, and 1% Triton-X100) solution. The first two washes were performed for 5 minutes each, while the third wash was extended to 30 minutes. During each wash, the beads were incubated on a rotating

wheel at 4°C and subsequently centrifuged for 2 minutes at 3000 x g to remove the supernatant. In the next step, HEK293 cells were lysed on ice as follows: cells were washed twice with 4 mL of 10 mM NEM (N-ethyl maleimide)-PBS (phosphate-buffered saline), then lysed for 10 minutes with 500 µL of lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA pH 8.0, 0.1% Triton-X100, 25 mM NEM, 5 mM NaF, 1 mM PMSF (phenylmethylsulfonyl fluoride), and EDTA-free protease inhibitor cocktail (resuspended according to the manufacturer's instructions, Sigma-Aldrich, S-8830). After lysing the cells, the cellular debris was separated from the cell extract by centrifuging at 10,000 x g for 10 minutes at 4°C. The resulting supernatant was collected. One-fifth of the cell extracts were collected for subsequent analysis. To capture the Flag-tagged SecPH proteins present in the cell extract, the previously prepared and washed Flag beads were incubated with the lysate on a rotating wheel at 4°C for 3 hours. During this incubation, the proteins of interest were bound to the Flag beads. Following the incubation, the bound proteins were extensively washed five times with freshly prepared ice-cold lysis buffer. Each wash involved the addition of 500 µL of lysis buffer, manual mixing of the beads, and subsequent centrifugation at 3000 x g for 3 minutes. This process ensured the removal of any non-specifically bound proteins. The bound proteins were eluted from the Flag beads in the final step using 2 mg/mL Flag peptides (Sigma Aldrich, F3290). The beads were incubated with Flag peptides on ice for 30 minutes, promoting the removal of the captured proteins from the beads. The eluted proteins were recovered by centrifugation for 2 minutes at 10,000 x g, and the resulting eluate was collected using a Hamilton syringe.

4. SDS-PAGE AND WESTERN BLOT

Proteins from the whole cell extracts and eluate obtained from the immunoprecipitation were separated based on their molecular weight using SDS-PAGE. The separated proteins were then transferred onto a PVDF membrane (Merck Millipore, IPVH00010) using a wet-transfer system (Bio-rad) at 100V and 4°C for a duration of 90 minutes. After the transfer, the membranes were blocked in a 5% non-fat milk solution prepared in Tris-buffered saline with 0.1% Tween-20 (TBS-T). This blocking step was carried out at room temperature for 1 hour. Subsequently, the milk was discarded, and primary antibodies were added to the membranes. The primary antibodies were diluted 5000 times in fresh TBS-T solution containing 5% non-fat milk. For the detection of SecPH Flag-tagged proteins, an anti-Flag primary antibody (Sigma-Aldrich, F3165) was used, and for VCP and LIMK2, an anti-HA primary antibody (Sigma-Aldrich, 3F10) was used. For evaluating inhibition, free SUMO2/3 proteins were

detected with an anti-SUMO2/3 (Abcam, ab81371) diluted 2000 times in a TBS-T solution containing 5% non-fat milk. Actin was included in the Western blot analysis to determine the quantity of proteins loaded onto the gel. To detect actin, an anti-actin primary antibody (Sigma-Aldrich, A1978) was used, which was diluted 10,000 times in a TBS-T solution containing 5% non-fat milk. The membranes were then incubated overnight at 4°C with the primary antibodies. After the incubation, the membranes were washed three times with TBS-T to remove any unbound antibodies. Following the washing, appropriate secondary antibodies conjugated to horseradish peroxidase were added to the membranes for the next detection step. Specifically, mouse secondary antibody (Invitrogen, 61-6520) for Flag-tagged protein, SUMO2/3, and actin or rat secondary antibody (Invitrogen, 62-9520) for HA-tagged protein was used, diluted 50000 times in TBS-T containing 5% non-fat milk. The membranes were incubated at room temperature for 2 hours with the secondary antibodies. Following the incubation, the membranes were washed three times in TBS-T for 20 minutes each to remove any excess antibodies. Finally, the proteins of interest were visualized using chemiluminescence (SuperSignalTM West Dura Extended Duration Substrate, PierceTM) and captured using the PXi imaging system (Syngene).

5. SUPPLEMENTARY MATERIAL

Table 1. List of plasmids used in this study.

NAME	ORIGIN
p3xFlag-Myc-CMV-24 - SecPH	Obtained from the team
p3xFlag-Myc-CMV-24 - SecPH - K1731R	Obtained from the team
p3xFlag-Myc-CMV-24 - SecPH - D1623G	Obtained from the team
p3xFlag-Myc-CMV-24 - SecPH - Y1587Δ	Obtained from the team
p3xFlag-Myc-CMV-24 - SecPH - R1748P	Obtained from the team
p3xFlag-Myc-CMV-24 - SecPH - R1748Q	Obtained from the team
p3xFlag-Myc-CMV-24 - SecPH - R1750E	Obtained from the team
p3xFlag-Myc-CMV-24 - SecPH - A1764S	Obtained from the team
p3xFlag-Myc-CMV-24 - SecPH - T1787M	Obtained from the team
pcDNA3 - 2xHA – LIMK2a	Obtained from the team
pcDNA3 - 2xHA – KIN	Obtained from the team
pcDNA3 - 2xHA – PDZ-SP	Obtained from the team
pcDNA3 - 2xHA – LIM	Obtained from the team
pEGFP-C1-VCP-EGFP-6xHIS-HA	Commercial (Addgene)
pEZ-MDH1-1xFLAG	Commercial (GeneCopoeia)
pcDNA3 – Ubc9 – SV5	Obtained from Ronald T. Hay
pcDNA3 – 6xHIS – SUMO2	Obtained from Ronald T. Hay

3. RESULTS

1. SUMOYLATION DOES NOT AFFECT THE INTERACTION BETWEEN VCP AND SecPH

In a previous study (Wang et al., 2011), VCP was identified as a novel binding partner of Nf1, sharing involvement in the same signaling pathway. The interaction was attributed to the leucine-rich domain (LRD) of Nf1, which contains 393 amino acids and includes SecPH and Heat-like repeat (HLR) domains (Figure 9).

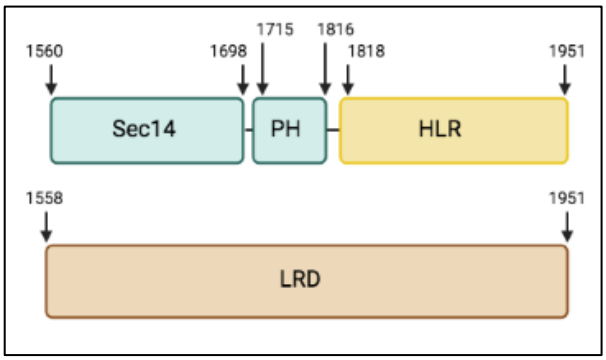

Figure 9. Comparison between the LRD domain and the SecPH domain. Arrows indicate the positions of the first and the last amino acid in each domain.

However, it is therefore unclear whether SecPH alone interacts with VCP, thus making it the first part of the experiment. Subsequently, the investigation proceeded to assess the effects of the K1731R substitution on this interaction. Additionally, the impact of K1731 SUMOylation was examined, as the substitution eliminates its SUMOylation potential. To address these questions, the interaction was examined under two distinct conditions: endogenous SUMOylation conditions, where proteins naturally undergo SUMOylation in cells and are modified by all SUMO proteins, and enhanced SUMOylation conditions achieved by overexpressing the Ubc9 conjugation enzyme and SUMO2 protein. When examining under endogenous SUMOylation conditions, HEK293 cells were transfected with plasmids expressing either Flag-tagged SecPH WT or SecPH K1731R mutant, along with VCP-EGFP-6xHis-HA. When examining under enhanced SUMOylation conditions, cells were additionally co-transfected with plasmids containing Ubc9 and SUMO2. Flag immunoprecipitation was performed, and Western blot analysis was conducted on both the cell lysate and eluates. To

assess the potential nonspecific binding of the VCP protein to the beads, a negative control was employed. In this control, cells were transfected only with the plasmid containing VCP. Additionally, to evaluate whether the conditions employed for Flag immunoprecipitation are effective in capturing the Flag-tagged protein, cells were transfected with the plasmid containing Flag-tagged Mdh1. If the conditions are indeed effective, it would be expected that Mdh1, being the target of the immunoprecipitation, would be captured and detected in the eluate. Alternatively, VCP, which is not supposed to bind to the beads, would not be present in the eluate. Anti-HA was used for detecting VCP and anti-Flag for detecting SecPH WT, SecPH K1731R mutant, and Mdh1. Based on the findings, several key observations can be made. Firstly, the negative control results indicate that VCP does not exhibit nonspecific binding to the beads, as it was not detected in the eluate. On the other hand, Mdh1, which was tagged with Flag, was successfully captured during the immunoprecipitation process (Figure 10). These findings confirm that the conditions used for capturing Flag-tagged proteins are suitable and effective.

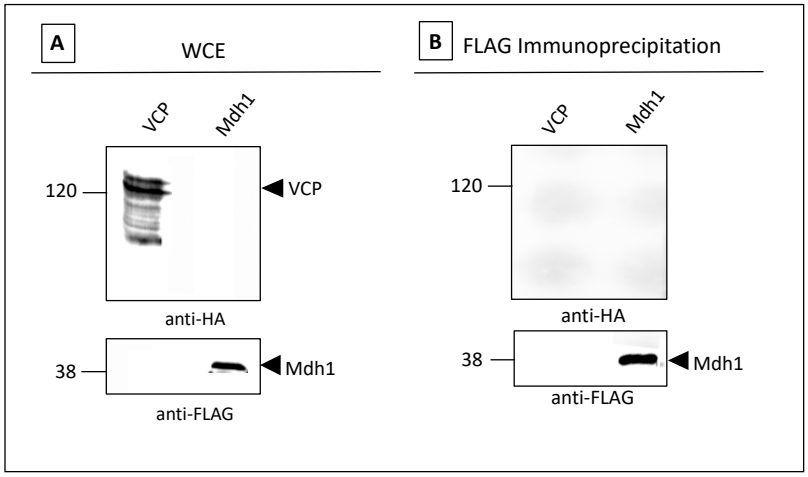

Figure 10. Negative control of the experiment and for VCP unspecific binding to Flag-tagged beads. Samples were separated on an 8% SDS-PAGE gel. **A.** Western blot analysis of the cell lysates before the Flag immunoprecipitation. **B.** Western blot analysis of the eluates after the Flag immunoprecipitation. Antibodies used for the Western blots are indicated under each picture. Arrows on the side, indicate the size of the different proteins revealed.

Secondly, it has been demonstrated that, under endogenous SUMOylation conditions, VCP exhibits an interaction not only with the LRD domain but also with the SecPH domain of Nf1. Furthermore, the K1731R mutation does not appear to impact this interaction. In the cell lysate,

both VCP and SecPH WT are expressed at similar levels, while the K1731R mutant shows higher expression, resulting in increased protein capture during Flag immunoprecipitation (Figure 11).

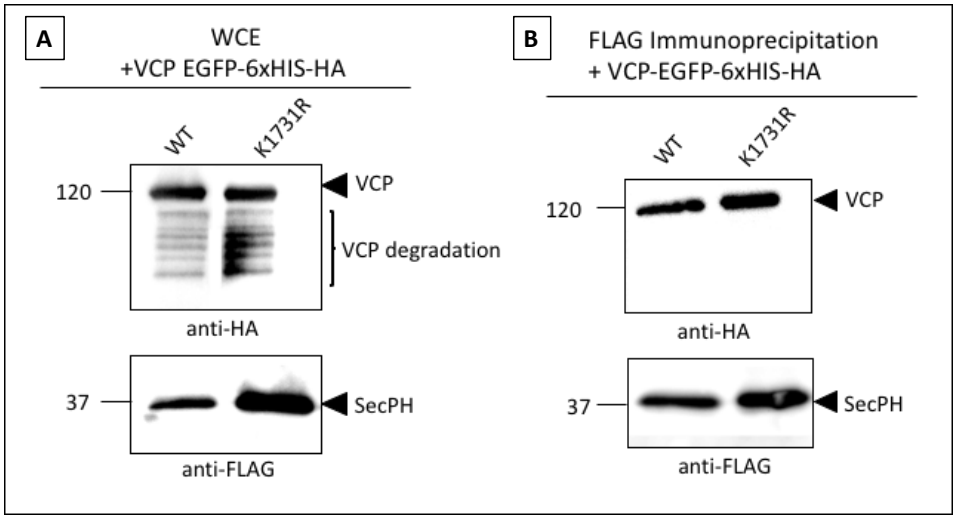

Figure 11. Interaction between SecPH WT and SecPH K1731R mutation with VCP under endogenous SUMOylation conditions. Samples were separated on an 8% SDS-PAGE gel. **A.** Western blot analysis of the cell lysates before the Flag immunoprecipitation. **B.** Western blot analysis of the eluates after the Flag immunoprecipitation. Antibodies used for the Western blots are indicated under each picture. Arrows on the side, indicate the size of the different proteins revealed.

Under enhanced SUMOylation conditions, where the K1731 lysine residue undergoes SUMOylation in SecPH WT but not in the K1731R mutant, the interaction between VCP and the K1731R mutant remains unaffected and resembles the interaction observed with SecPH WT (Figure 12).

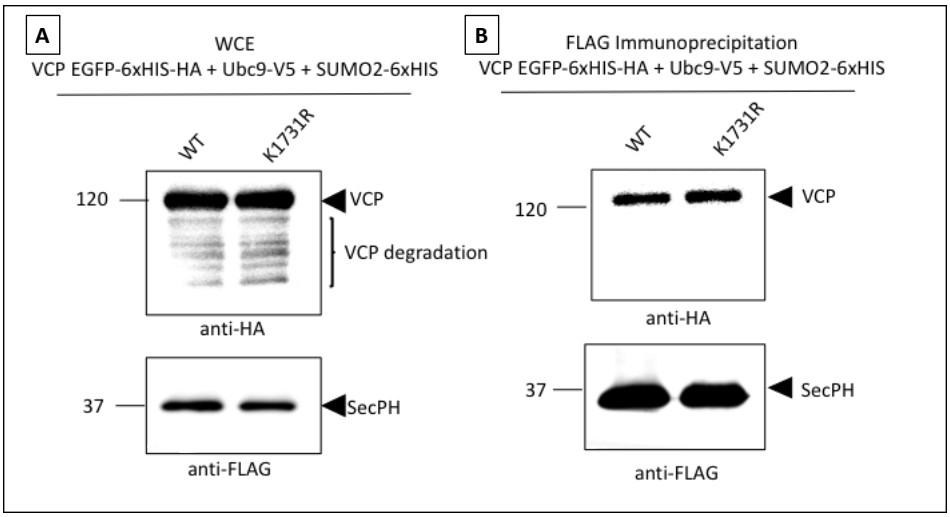

Figure 12. Interaction between SecPH WT and SecPH K1731R mutant with VCP under enhanced SUMOylation conditions. Samples were separated on an 8% SDS-PAGE gel. **A.** Western blot analysis of the cell lysates before the Flag immunoprecipitation. **B.** Western blot analysis of the eluates after the Flag immunoprecipitation. Antibodies used for the Western blots are indicated under each picture. Arrows on the side, indicate the size of the different proteins revealed.

2. K1731R MUTANTION WEAKENS THE INTERACTION WITH LIMK2 IN ENDOGENOUS SUMOYLATION CONDITIONS

To explore the interaction of SecPH with LIMK2, a similar experimental approach as the one used for studying the VCP interaction was employed. HEK293 cells were transfected with the LIMK2 containing plasmid, tagged with two HA-tags on its N-terminal end, and Flag-tagged SecPH WT or SecPH K1731R plasmid. Alongside these plasmids, the necessary plasmids for investigating the interaction under enhanced SUMOylation conditions were introduced (plasmids containing Ubc9 and SUMO2). Following transfection, Flag immunoprecipitation was performed to capture the formed complexes and both the cell lysate and eluate were subjected to Western blot analysis. On the Western blot, LIMK2 was detected using HA-antibody, while the WT and K1731R SecPH mutant was detected with Flag-antibody. Analysis of the Western blot results revealed an interesting finding. In previous experiments conducted under endogenous conditions, the team successfully demonstrated the interaction between SecPH WT and LIMK2. This interaction was reproduced in the current study as well. Upon examining the SecPH variant carrying the K1731R mutation, it was

observed that the interaction with LIMK2 appeared to be weaker compared to the interaction with SecPH WT under endogenous conditions (Figure 13). It is important to note that this specific interaction has not been previously tested. Indeed, this effect might be due to SUMOylation which is prevented by the K1731R mutation.

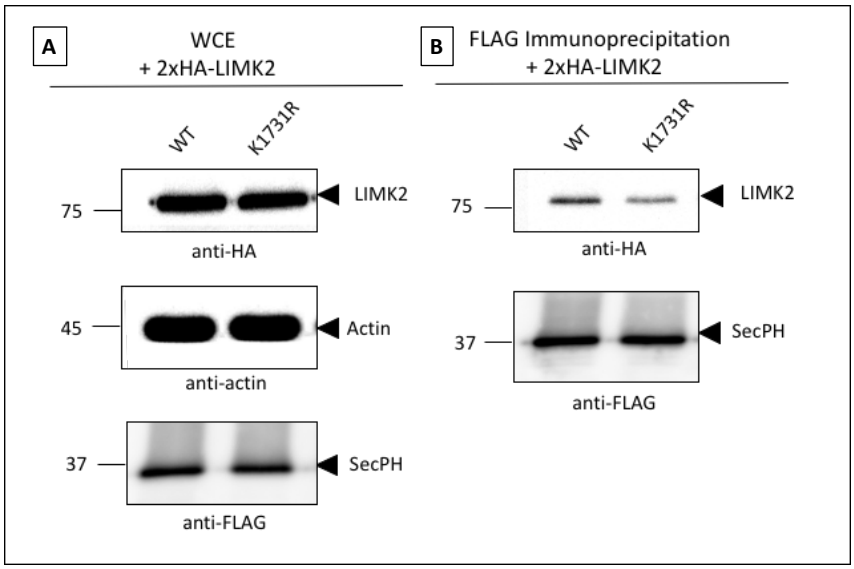

Figure 13. Interaction between SecPH WT and SecPH K1731R mutant with LIMK2 under endogenous SUMOylation conditions. Samples were separated on an 8% SDS-PAGE gel. **A.** Western blot analysis of the cell lysates before the Flag immunoprecipitation. **B.** Western blot analysis of the eluates after the Flag immunoprecipitation. Antibodies used for the Western blots are indicated under each picture. Arrows on the side, indicate the size of the different proteins revealed. Actin is used as a control to normalize proteins level.

According to this hypothesis, SUMOylation of K1731 might enhance the interaction of SecPH with LIMK2. However, when investigating the impact of SUMOylation on this interaction, no noticeable difference was observed in the interaction with LIMK2 between the SecPH WT and K1731R mutant (Figure 14). If SUMOylation was involved, we would expect the opposite and an even bigger difference between SecPH WT and SecPH K1731R for the interaction with LIMK2.

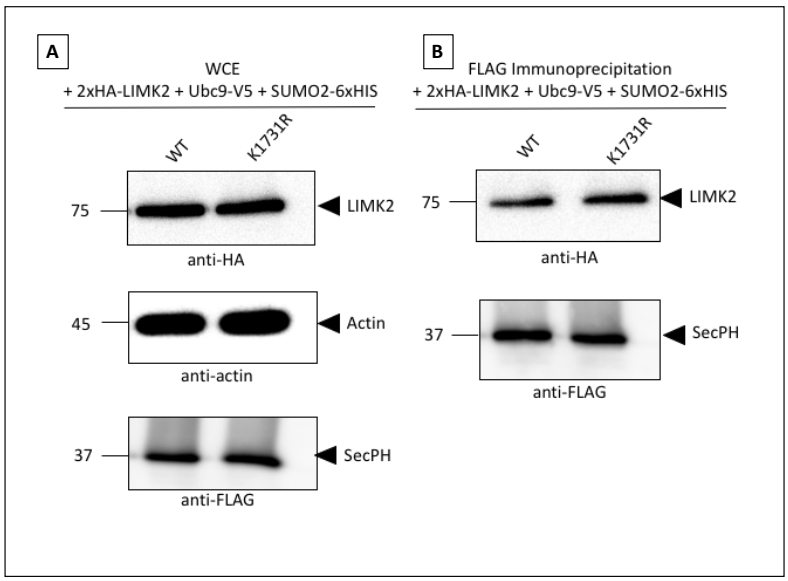

Figure 14. Interaction between SecPH WT and SecPH K1731R mutant with LIMK2 under enhanced SUMOylation conditions. Samples were separated on an 8% SDS-PAGE gel. **A.** Western blot analysis of the cell lysates before the Flag immunoprecipitation. **B.** Western blot analysis of the eluates after the Flag immunoprecipitation. Antibodies used for the Western blots are indicated under each picture. Arrows on the side, indicate the size of the different proteins revealed. Actin is used as a control to normalize proteins level.

To further explore the influence of SUMOylation on this interaction, an additional experiment was conducted by using a commercially available inhibitor called ML792 to inhibit the SUMOylation pathway. Specifically, the SAE1/SAE2 activating enzymes were inhibited. The efficacy of the inhibitor was confirmed through Western blot analysis, using an anti-SUMO2/SUMO3 antibody to detect the levels of free SUMO2/SUMO3 proteins in the cell lysate on a 15% SDS-PAGE gel (Figure 15A). Comparing the samples treated with the inhibitor to the non-treated samples, it is evident that the concentration of free SUMO2/SUMO3 proteins is significantly higher in the inhibitor-treated samples. This outcome comes from the inhibition of the conjugation process, which prevents SUMO proteins from attaching to their target proteins, thereby resulting in an accumulation of non-attached SUMO proteins within the cells. The results presented in Figure 15B, show that the SecPH WT and the SecPH K1731R mutant interact equally with LIMK2, therefore the interaction is SUMOylation-dependant.

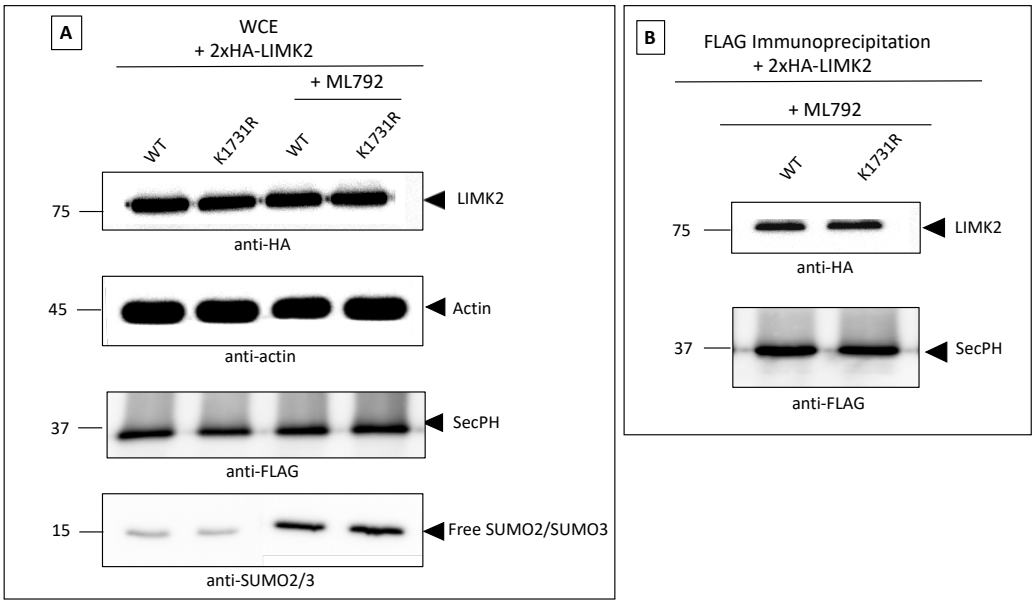

Figure 15. Interaction between SecPH WT and SecPH K1731R mutant with LIMK2 in conditions when SUMOylation is inhibited. Samples were separated on an 8% and 15% SDS-PAGE gel. **A.** Western blot analysis of the treated and untreated cell lysate before Flag immunoprecipitation. To determine if the inhibition of SUMOylation was successful, SUMO2/SUMO3 proteins were detected with an anti-SUMO2/SUMO3 antibody. **B.** Western blot analysis of eluates after Flag immunoprecipitation. Antibodies used for the Western blots are indicated under each picture. Arrows on the side, indicate the size of the different proteins revealed. Actin is used as a control to normalize proteins level.

The Flag immunoprecipitation experiment in endogenous conditions was repeated three times for SecPH WT and K1731R mutant. The bands detected in the Western blots with anti-HA and anti-Flag antibodies were measured to calculate the HA-LIMK2/FLAG-SecPH ratio (Figure 16).

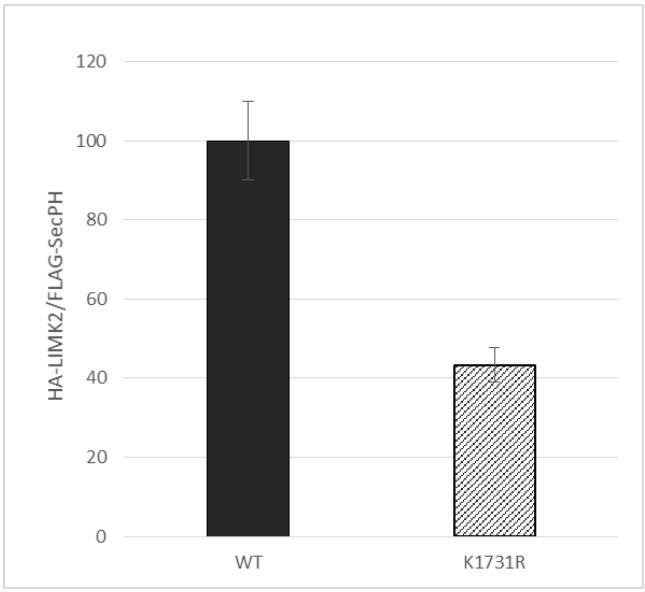

Figure 16. The difference in the interaction with LIMK2 between SecPH WT and SecPH K1731R mutant. HA-LIMK2/FLAG-SecPH ratio was calculated after quantification of Western blots by densitometry using ImageLab software by BioRad.

3. K1731R MUTATION SEEMS TO WEAKEN THE INTERACTION WITH THE KIN DOMAIN OF LIMK2 IN ENDOGENOUS SUMOYLATION CONDITIONS

In 2012., Valle et al. identified, alongside the entire LIMK2, domains which are necessary for the interaction with the SecPH domain under endogenous SUMOylation conditions (Figure 17). These domains are KIN and PDZ-SP. Since the interaction with the K1731R mutants has never been tested before, in order to get a detailed insight into this interaction, Flag immunoprecipitation experiments were conducted.

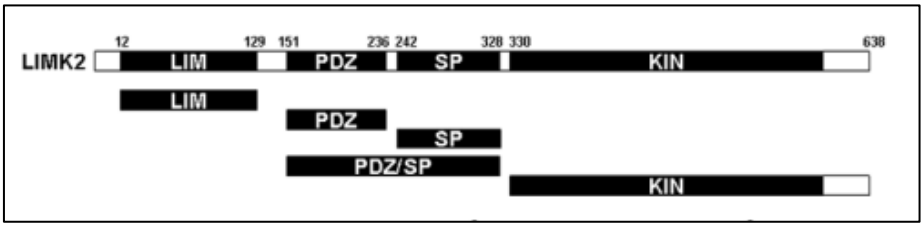

Figure 17. Domains of LIMK2. The KIN and PDZ-SP domains interact with SecPH. Source : Vallée et al., 2012

HEK293 cells were transfected with plasmids containing individual domains of LIMK2, KIN, or PDZ-SP, each tagged with two HA-tags, in addition to the Flag-tagged SecPH-derived plasmids. To capture the complexes formed by these interacting proteins, Flag immunoprecipitation was conducted. As a negative control, cells were transfected with a plasmid containing the LIM domain tagged with two HA-tags, which is known to lack interaction with SecPH. The results presented in Figure 18B reveal that the SecPH K1731R mutant interacts with the KIN and PDZ-SP domains. However, the interaction appears to be weaker with the KIN domain compared to the interaction observed with SecPH WT. It is important to note that the experiment will need to be repeated to determine the reproducibility and significance of this result.

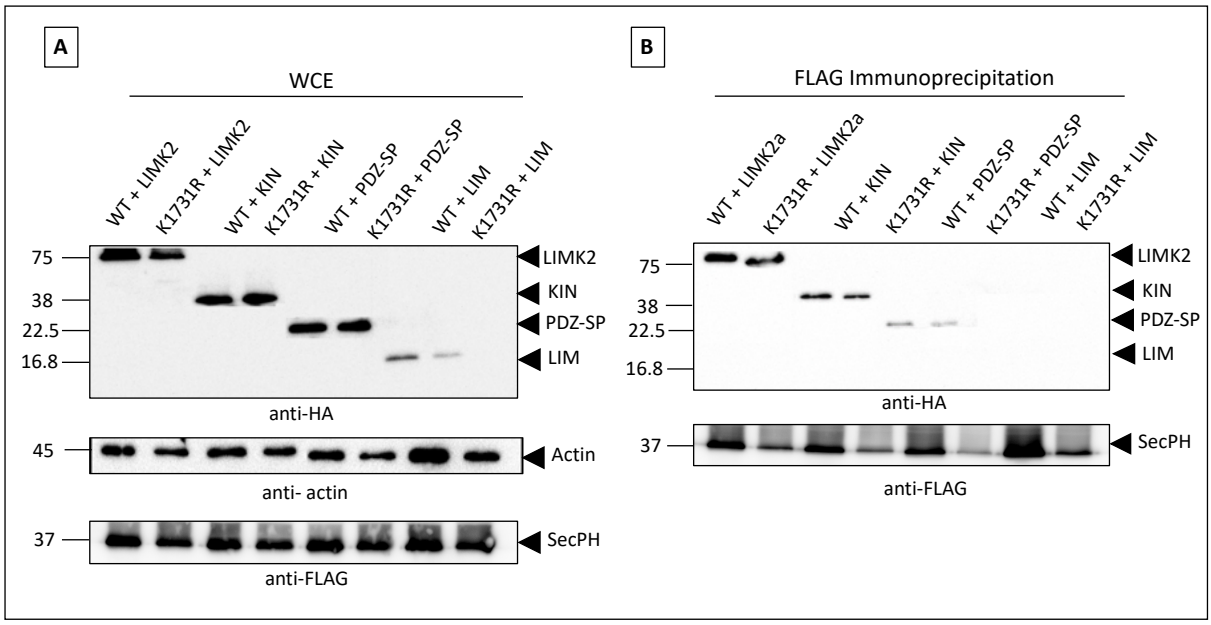

Figure 18. Interaction between SecPH WT and K1731R mutant and LIMK2 domains. Samples were separated on a 15% SDS-PAGE gel. **A.** Western blot analysis of cell lysate before Flag immunoprecipitation. **B.** Western blot analysis of eluates after Flag immunoprecipitation. Antibodies used for the Western blots are indicated under each picture. Arrows on the side, indicate the size of the different proteins revealed. Actin is used as a control to normalize proteins level.

4. R1748P AND K1750E PATHOGENIC MUTATIONS WHICH AFFECT K1731 SUMOYLATION WEAKEN THE INTERACTIONS WITH LIMK2 IN ENDOGENOUS SUMOYLATION CONDITIONS

To investigate the interaction of different missense SecPH pathogenic mutants with LIMK2, various mutants which affect functionally important domains of the SecPH as well as K1731 SUMOylation were used: R1748P, R1748Q, and K1750E located in the β-protrusion region, D1623G located in the Sec14 module and A1764S and T1787M, which are exposed on the surface and are located in the PH module. The Y1587Δ was recently constructed by the team and is also localized in the Sec14 module. The experiment was conducted by transfecting HEK293 cells with Flag-tagged SecPH-derived plasmids containing each mutant individually, along with the HA-tagged LIMK2 plasmid. Flag immunoprecipitation was performed to capture the interacting complexes and both cell lysate and eluate were analyzed by Western blot. Notable findings can be observed from the results depicted in Figure 18. Firstly, the expression levels of LIMK2 and SecPH proteins appear to be similar, except for the SecPH D1623G and Y1587Δ mutants, which exhibit lower expression levels (Figure 19A). Notably, the behavior of the Y1587Δ mutant, which has not been previously studied, appears to resemble that of the D1623G mutant. Secondly, the R1748P and K1750E mutants display weaker interactions with LIMK2 compared to the other mutants (Figure 19B). It is worth mentioning that both of these mutants, along with R1748Q, are located in the β-protrusion region of SecPH and all three mutations affect the SUMOylation of K1731. However, despite the shared impact on SUMOylation, the mutants exhibit different interaction patterns. This suggests that K1731 SUMOylation not only plays a role in the interaction but also indicates the requirement for a specific structural conformation for the interaction.

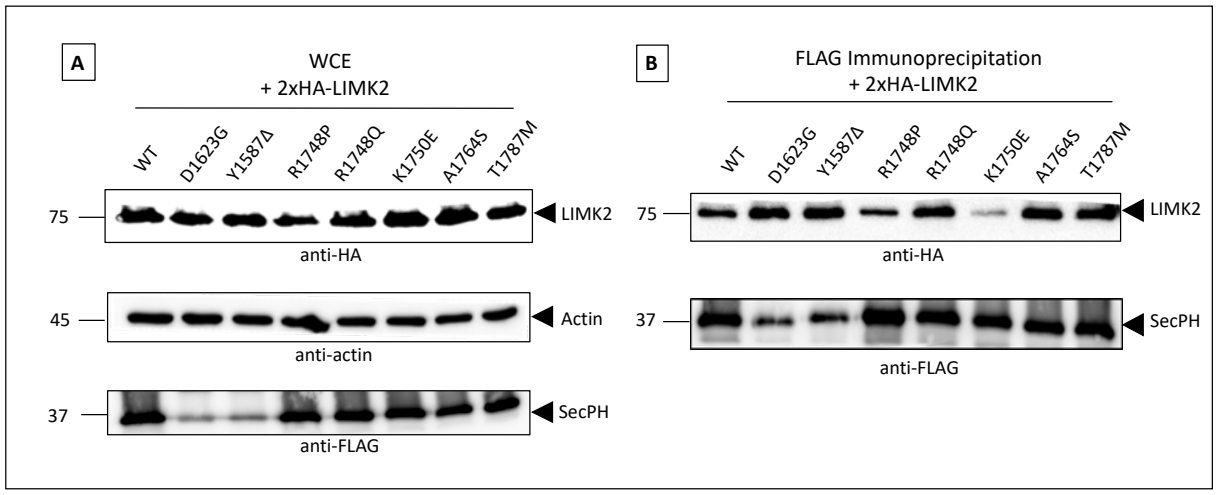

Figure 19. Interaction between LIMK2 and different SecPH pathogenic mutants in endogenous SUMOylation conditions. Samples were separated on an 8% SDS-PAGE gel. **A.** Western blot analysis of the cell lysate before Flag immunoprecipitation. **B.** Western blot analysis of eluates after Flag immunoprecipitation. Antibodies used for the Western blots are indicated under each picture. Arrows on the side, indicate the size of the different proteins revealed. Actin is used as a control to normalize proteins level.

4. DISCUSSION AND CONCLUSIONS

4.1. DISCUSSION

The research team "Cell Signalling and Neurofibromatosis" recently demonstrated that the K1731 lysine residue within the SecPH domain of Nf1 undergoes SUMOylation. Even though the K1731R mutation, which prevents this SUMOylation, is pathogenic, it does not impact the Ras-GAP activity of Nf1. This led to speculation that the mutation might affect the interaction of the SecPH domain with various binding partners. Additionally, considering the structural similarity between arginine and lysine, the hypothesis is that SUMOylation might play a role. To investigate the influence of SUMOylation, we initiated a comparative analysis to examine the interactions between SecPH WT and SecPH K1731R with two partners, VCP and LIMK2, under three distinct conditions: natural endogenous SUMOylation, enhanced SUMOylation, and in the absence of SUMOylation.

In a previous study by Wang et al. in 2011, VCP was identified as a novel binding partner of Nf1. They characterized the interaction between VCP's D1D2 region on the C-terminus and the larger leucine-rich domain (LRD) of Nf1, which includes the SecPH domain. In our study, our focus was specifically on investigating the interaction between VCP and the

SecPH domain of Nf1, as well as exploring the role of K1731 SUMOylation in this interaction. Our results indicate that the SecPH domain does indeed interact with VCP. Interestingly, we did not observe a noticeable difference in the interaction between SecPH WT and SecPH K1731R with VCP. This suggests that SUMOylation of K1731 may not significantly impact the SecPH-VCP interaction. However, it is important to note that VCP itself has been reported to undergo SUMOylation on its N-terminus by SUMO1 (Wang et al. 2016). Considering the proximity of the N-terminus to the D1D2 domain, it is possible that N-terminus SUMOylation could influence the interactions involving the D1D2 domain. Although our study focused on SUMOylation by SUMO2, it is worth considering that SUMO2 may also play a role in VCP SUMOylation and could potentially modify the SecPH-VCP interaction. Additionally, since both proteins are SUMOylated, it is challenging to observe clear differences when Ubc9 and SUMO2 are overexpressed to enhance SUMOylation within cells. To overcome this challenge and assess the importance of SecPH SUMOylation in the SecPH-VCP interaction, a possible approach that could be effective is to work with HEK293 cells that express only one partner (VCP), while manipulating SUMOylation enhancement or inhibition exclusively in cells expressing the SecPH derivatives. Subsequently, Flag immunoprecipitations could be performed after mixing the cell lysates from both conditions to investigate the interaction between the two partners.

The analysis of the second SecPH interaction with LIMK2 revealed interesting findings. Under endogenous SUMOylation conditions, our results demonstrated that the SecPH K1731R mutant exhibited a weaker interaction with LIMK2 compared to the SecPH WT. This observation was reproducible across three repeated experiments. Two potential hypotheses were proposed to explain this difference: 1) K1731 SUMOylation plays a role in enhancing the interaction between SecPH and LIMK2, and 2) Subtle and unexpected structural differences between SecPH K1731R and SecPH WT contribute to this discrepancy. To further investigate these hypotheses, we examined the interaction under enhanced SUMOylation conditions. However, the results obtained were more challenging to interpret and did not align with either of the two hypotheses. This prompted us to explore the effects of inhibiting SUMOylation. Surprisingly, the interaction between LIMK2 and both the SecPH WT and SecPH K1731R mutant appeared to be similar to each other, which could support the first hypothesis regarding the role of K1731 SUMOylation in the SecPH-LIMK2 interaction. However, the inhibition of SUMOylation enhanced both interactions, contradicting the initial hypothesis. This suggests the involvement of other factors in explaining these observed behaviors. Although LIMK2 has not been previously identified as being SUMOylated, our analysis of its sequence using

SUMOylation prediction software revealed potential SUMOylation consensus sequences. Therefore, the hard-to-interpret results we obtained may be influenced by LIMK2 SUMOylation. As mentioned earlier regarding VCP, conducting new experiments involving cells that produce only one partner at a time and subsequently combining their lysates before immunoprecipitation could help provide further clarity on the role of K1731 SUMOylation in the SecPH-LIMK2 interaction. Additionally, our findings revealed that under endogenous SUMOylation conditions, the K1731R mutation seemed to weaken the interaction between SecPH and the KIN domain of LIMK2, while the interaction with the PDZ-SP domain appeared unaffected. However, it is important to note that further confirmation through repetition of the experiment is required to validate these results.

To further investigate the role of K1731 SUMOylation in the interaction with LIMK2, we examined various pathogenic mutants within the SecPH domain (Figure 20), each displaying distinct SUMOylation profiles. Surprisingly, our results demonstrated that the R1748P and K1750E mutants exhibited weaker interactions with LIMK2 compared to the other mutants. Interestingly, three pathogenic mutations (R1748P, R1748Q, and K1750E), which are located in the exposed β-protrusion of SecPH, have been previously shown to abolish K1731 SUMOylation. If K1731 SUMOylation played a crucial role in the interaction, we would expect all three mutants to exhibit similar behavior in their interaction with LIMK2. However, our observations indicated that different mutations yielded different outcomes, suggesting the involvement of additional factors. One hypothesis is that the R1748P and K1750E mutations profoundly affect the conformation and properties of the β-protrusion. The substitution of arginine with proline (R1748P), a rigid residue known to induce turn and loop structures, is more detrimental than the substitution with glutamine (R1748Q), a polar uncharged amino acid. Moreover, the substitution of K1750 with glutamic acid (K1750E) completely changes the charge, resulting in significant alterations in the surface properties of the β-protrusion. These findings highlight the complexity of the interaction and suggest that factors beyond K1731 SUMOylation may play a critical role in modulating this interaction. Further investigations are needed to unravel the precise mechanisms of how these mutations affect the conformation and properties of the β-protrusion and their impact on the interaction with LIMK2.

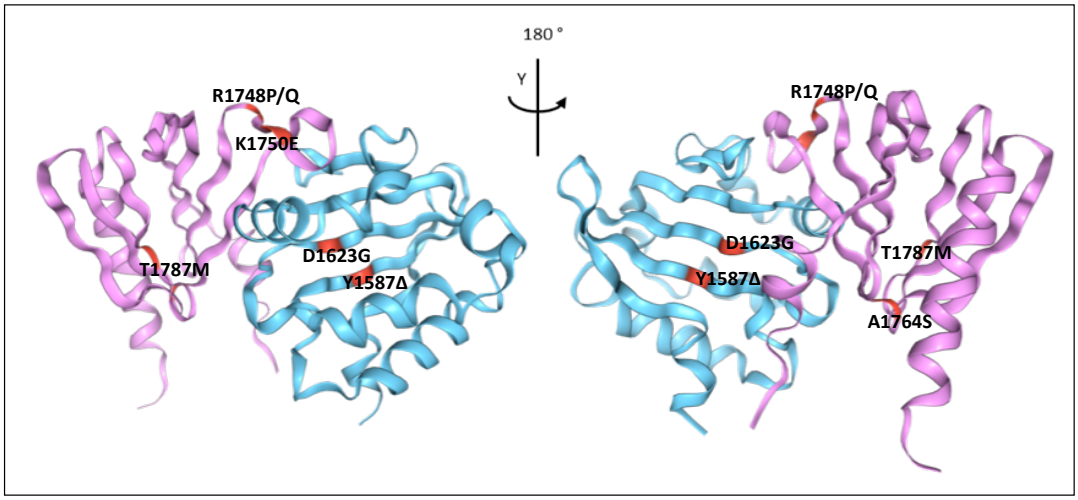

Figure 20. Localization of the mutations on the SecPH 3D structure. The Sec14 module is colored blue, the PH module pink, and the location of mutations red.

Our findings, therefore, underscore the significance of the β-protrusion in the SecPH domain for its interaction with LIMK2. This observation aligns with a previous study conducted by Deraredj et al. in 2016, which demonstrated that substituting the same residues (R1748 and K1750) with alanine or deleting K1750 affected the interaction with the serotonin 5-hydroxytryptamine 6 receptor (5-HT6), one of known SecPH binding partners. Thus, our results further emphasize the critical role of the β-protrusion conformation in mediating the interactions between SecPH and its binding partners. On the other hand, mutations within the PH module, such as A1764S and T1787M, which exhibit a wild-type SUMOylation profile, did not seem to impact the interaction with LIMK2, nor were they involved in the PH-5HT6 interaction.

Furthermore, it is noteworthy that the Y1587Δ mutant, a newly constructed variant in the laboratory, has never been tested before and demonstrates similar behavior to the unstable D1623G mutant. These two residues are located on the Sec14 module of the SecPH domain and are located in close proximity. Based on the 3D structure, there is a hypothesis that they establish an interchain hydrogen bond. This implies that they may share similar characteristics in terms of their pathogenicity, causing instability in the protein when subjected to mutation, and possibly even exhibiting the same SUMOylation profile. Nevertheless, additional research on the full-length Nf1 is required to validate all of these findings

4.2. FUTURE PERSPECTIVE

The differential interactions observed between LIMK2 and various pathogenic mutants in SecPH have revealed exciting findings for further investigation. As SecPH plays a crucial role in regulating the Rho/ROCK/LIMK2/cofilin pathway, understanding the impact of these interactions through specific functional assays can greatly enhance our comprehension of disease development. The interaction can be studied in detail and investigated by using specific functional studies that focus on detecting LIMK2 phosphorylation by ROCK or by assessing the formation of stress fibers in HeLa cell lines. These approaches allow for a detailed examination of the specific mechanisms involved in this interaction.

However, the current experimental conditions for assessing the effect of SUMOylation may yield varied outcomes due to the possibility of multiple proteins being SUMOylated, thereby influencing the interactions. To overcome this limitation, a more focused approach will be adopted. By overexpressing SecPH, Ubc9, and SUMO2 in one set of cells, selective SUMOylation of SecPH can be achieved, while the potential binding partners are expressed in separate cells. Subsequently, immunoprecipitation can be performed by mixing the lysates, enabling the isolation of interacting proteins. Employing such an experimental design ensures a more precise analysis of the interactions without unwanted SUMOylation events.

Furthermore, these investigations have also highlighted the possibility of LIMK2 being a target for SUMOylation. This revelation opens up exciting doors for further research into the intricate interplay between LIMK2 and the SUMOylation machinery, providing new insights into the regulatory mechanisms governing LIMK2's functions.

4.3. CONCLUSION

In conclusion, the interaction experiments revealed distinct interactions between SecPH pathogenic mutants, which affect K1731 SUMOylation, and LIMK2. While we did not establish a direct involvement of SUMOylation in the SecPH-LIMK2 interaction, our findings demonstrated that various mutants located in the β-protrusion region exhibited weaker interaction with LIMK2. This highlights the crucial significance of the β-protrusion and its conformation in forming interactions with diverse binding partners.

5. LITERATURE

Anastasaki, C., Orozco, P., & Gutmann, D. H. (2022). RAS and beyond: the many faces of the neurofibromatosis type 1 protein. *DMM Disease Models and Mechanisms*, **15**(2).

Bergoug, M., Doudeau, M., Godin, F., Mosrin, C., Vallée, B., & Bénédetti, H. (2020). Neurofibromin Structure, Functions and Regulation. *Cells*, **9**(11)

Chang, H. M., & Yeh, E. T. H. (2020). Sumo: From bench to bedside. *Physiological Reviews*, **100**(4), 1599–1619.

Cichowski, K., Santiago, S., Jardim, M., Johnson, B. W., & Jacks, T. (2003). Dynamic regulation of the Ras pathway via proteolysis of the NF1 tumor suppressor. *Genes and Development*, **17**(4), 449–454.

Cimino, P. J., & Gutmann, D. H. (2018). Neurofibromatosis type 1. In *Handbook of Clinical Neurology*, Vol. 148, Elsevier B.V., pp. 799–811.

D'Angelo, I., Welti, S., Bonneau, F., & Scheffzek, K. (2006). A novel bipartite phospholipid-binding module in the neurofibromatosis type 1 protein. *EMBO Reports*, **7**(2), 174–179.

Dreveny, I., Pye, V. E., Beuron, F., Briggs L., Isaacson R., Matthews S., Mckeown C., Yuan X., Zhang X. & Freemont, P. S. (2004). p97 and close encounters of every kind: a brief review. *Biochemical Society Transactions,* **32**(5), 715-720.

Feng, L., Yunoue, S., Tokuo, H., Ozawa T., Zhang D., Patrakitkomjorn S., Ichimura T., Saya H. & Araki, N. (2004). PKA phosphorylation and 14-3-3 interaction regulate the function of neurofibromatosis type I tumor suppressor, neurofibromin. *FEBS Letters*, **557**(1–3), 275–282.

Halawani, D., & Latterich, M. (2006). p97: The Cell's Molecular Purgatory? *Molecular Cell*, **22**(6), 713–717.

Han, Z. J., Feng, Y. H., Gu, B. H., Li, Y. M., & Chen, H. (2018). The post-Translational modification, SUMOylation, and cancer (Review). *International Journal of Oncology*, **52**(4), 1081–1094.

Hendriks, I. A., Lyon, D., Young, C., Jensen, L. J., Vertegaal, A. C. O., & Nielsen, M. L. (2017). Site-specific mapping of the human SUMO proteome reveals co-modification with phosphorylation. *Nature Structural and Molecular Biology*, **24**(3), 325–336.

Jordan, M., & Wurm, F. (2004). Transfection of adherent and suspended cells by calcium phosphate. *Methods*, **33**(2), 136–143.

K, S. T., Joshi, G., Arya, P., Mahajan, V., Chaturvedi, A., & Mishra, R. K. (2021). SUMO and SUMOylation Pathway at the Forefront of Host Immune Response. *Frontiers in Cell and Developmental Biology*, **9**

Liang, Y. C., Lee, C. C., Yao, Y. L., Lai, C. C., Schmitz, M. L., & Yang, W. M. (2016). SUMO5, a novel poly-SUMO isoform, regulates PML nuclear bodies. *Scientific Reports*, **6**

Mukhopadhyay, D., & Dasso, M. (2007). Modification in reverse: the SUMO proteases. *Trends in Biochemical Sciences*, **36**(2), 286–295.

Peltonen, S., Kallionpää, R. A., & Peltonen, J. (2017). Neurofibromatosis type 1 (NF1) gene: Beyond café au lait spots and dermal neurofibromas. *Experimental Dermatology*, **26**(7), 645–648.

Rassmusen, S. A., & Friedman, J. M. (2000). NF1 gene and neurofibromatosis 1. *American Journal of Epidemiology*, **151**(1), 33–40.

Scott, R. W., & Olson, M. F. (2007, June). LIM kinases: Function, regulation and association with human disease. *Journal of Molecular Medicine*, **85**(6), 555–568.

Shen, M. H., Harper, P. S., Upadhyaya, M., Shen, M. H., Harper, P. S., & Upadhyaya, M. (1996). Molecular genetics of neurofibromatosis type 1 (NFl). *Journal of medical genetics*, **33**(1), 2–17.

Starinsky-Elbaz, S., Faigenbloom, L., Friedman, E., Stein, R., & Kloog, Y. (2009). The pre-GAP-related domain of neurofibromin regulates cell migration through the LIM kinase/cofilin pathway. *Molecular and Cellular Neuroscience*, **42**(4), 278–287.

Vallée, B., Doudeau, M., Godin, F., Gombault, A., Tchalikian, A., de Tauzia, M. L., & Bénédetti, H. (2012). Nf1 RasGAP Inhibition of LIMK2 Mediates a New Cross-Talk between Ras and Rho Pathways. *PLoS ONE*, **7**(10).

Wang, L., Qian, J., Yang, Y., & Gu, C. (2021). Novel insights into the impact of the SUMOylation pathway in hematological malignancies (Review). *International Journal of Oncology*, **59**(3), 73.

Wang, T., Xu, W., Qin, M., Yang, Y., Bao, P., Shen, F., Zhang, Z., & Xu, J. (2016). Pathogenic mutations in the valosin-containing protein/p97(VCP) N-domain inhibit the SUMOylation of VCP and lead to impaired stress response. *Journal of Biological Chemistry*, **291**(27), 14373–14384.

Watts, G. D., Wymer, J., Kovach, M. J., Mehta, S. G., Mumm, S., Darvish, D., Pestronk, A., Whyte, M. P., & Kimonis, V. E. (2004). Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. *Nature Genetics*, **36**(4), 377–381.

Yuan, Y., Gaither, K., Kim, E., Liu, E., Hu, M., Lengel, K., Qian, D., Xu, Y., Wang, B., Knipprath, H., & Liu, D. X. (2018). SUMO2/3 modification of activating transcription factor 5 (ATF5) controls its dynamic translocation at the centrosome. *Journal of Biological Chemistry*, **293**(8), 2839–2848.

Zhong, Q., Xiao, X., Qiu, Y., Xu, Z., Chen, C., Chong, B., Zhao, X., Hai, S., Li, S., An, Z., & Dai, L. (2023). Protein posttranslational modifications in health and diseases: Functions, regulatory mechanisms, and therapeutic implications. *MedComm*, **4**(3)

ABSTRACT

Neurofibromatosis type I (NF1) is a genetic disease caused by mutations in the *NF1* gene, leading to the aberrant function of the tumor-suppressor protein neurofibromin (Nf1). Nf1 regulates Ras activity through its GRD (GAP-related domain) domain and participates in various signaling pathways via its SecPH domain, involved in lipid and protein interactions. Recently, SUMOylation, a rare post-translational modification, has been discovered on Nf1. At the molecular level, SUMOylation can modulate the activity, stability, localization, and interaction pattern of target proteins. Studies on Nf1 SUMOylation have identified a highly conserved and surface-exposed lysine residue (K1731) in the SecPH domain, demonstrating the importance of SecPH SUMOylation in an unidentified function of Nf1. Pathogenic variants of Nf1 with neurofibromatosis have been found to harbor a substitution of this SUMO acceptor and show a different SUMOylation profile. However, the GTPase activity of this Nf1 variant is unaffected, leading to the hypothesis that SUMOylation of Nf1 could regulate its interaction with partners. This study examines the impact of SUMOylation on the interaction between the SecPH domain of Nf1 and its interacting partners, LIMK2, a key kinase in the Rho/ROCK/LIMK2/cofilin pathway, and VCP/p97, versatile AAA (ATPase associated with a variety of cellular activities) protein. Three experimental conditions were employed. First, endogenous SUMOylation was evaluated using wild-type and SUMOylation-deficient (K1731R) Flag-SecPH mutants in HEK293 cells. LIMK2 and its domains were co-transfected. The second condition assessed enhanced SUMOylation by co-transfecting cells with Ubc9 and SUMO2 plasmids. Lastly, the impact of total SUMOylation inhibition was assessed using a SUMOylation inhibitor. Immunoprecipitation assays with Flag beads and subsequent western blot analysis were performed using tag-specific antibodies. Results indicate that SUMOylation does not play a role in the SecPH-VCP interaction. However, for the SecPH-LIMK2 interaction, differential affinities were observed between the SUMOylation-deficient SecPH mutant and LIMK2 in the presence of endogenous SUMOylation. Additionally, the interaction was affected by pathogenic variants in the SecPH domain, indicating the significance of the β-protrusion region in this interaction.

Keywords: neurofibromatosis type I, LIMK2, VCP, SUMOylation, K1731, cell signalling

RÉSUMÉ SCIENTIFIQUE

La neurofibromatose de type I (NF1) est une maladie génétique causée par des mutations dans le gène *NF1*, ce qui entraîne une fonction anormale de la protéine suppresseur de tumeur neurofibromine (Nf1). Nf1 régule l'activité de Ras à travers son domaine GRD (GAP-related domain) et participe à diverses voies de signalisation via son domaine SecPH, impliqué dans les interactions lipidiques et protéiques. Récemment, la SUMOylation, une modification post-traductionnelle rare, a été découverte sur Nf1. Au niveau moléculaire, la SUMOylation peut moduler l'activité, la stabilité, la localisation et le schéma d'interaction des protéines cibles. Des études sur la SUMOylation de Nf1 ont identifié un résidu lysine hautement conservé et exposé en surface (K1731) dans le domaine SecPH, démontrant l'importance de la SUMOylation de SecPH dans une fonction non identifiée de Nf1. Des variants pathogènes de Nf1 associés à la neurofibromatose ont été trouvés porteurs d'une substitution de cet accepteur SUMO et montrent un profil de SUMOylation différent. Cependant, l'activité GTPase de ce variant Nf1 n'est pas affectée, ce qui conduit à l'hypothèse que la SUMOylation de Nf1 pourrait réguler son interaction avec ses partenaires. Cette étude examine l'impact de la SUMOylation sur l'interaction entre le domaine SecPH de Nf1 et ses partenaires interactifs, LIMK2, une kinase clé dans la voie Rho/ROCK/LIMK2/cofiline, et VCP/p97, une protéine AAA polyvalente (ATPase associée à diverses activités cellulaires). Trois conditions expérimentales ont été utilisées. Tout d'abord, la SUMOylation endogène a été évaluée en utilisant des mutants Flag-SecPH de type sauvage et déficients en SUMOylation (K1731R) dans des cellules HEK293. LIMK2 et ses domaines ont été co-transfectés. La deuxième condition a évalué la SUMOylation renforcée en co-transfectant les cellules avec des plasmides Ubc9 et SUMO2. Enfin, l'impact de l'inhibition totale de la SUMOylation a été évalué en utilisant un inhibiteur de SUMOylation. Des tests d'immunoprécipitation avec des billes Flag et une analyse ultérieure par western blot ont été réalisés en utilisant des anticorps spécifiques. Les résultats indiquent que la SUMOylation ne joue pas de rôle dans l'interaction SecPH-VCP. Cependant, pour l'interaction SecPH-LIMK2, des affinités différentielles ont été observées entre le mutant SecPH déficient en SUMOylation et LIMK2 en présence de SUMOylation endogène. De plus, l'interaction est affectée pour des variants pathogènes dans le domaine SecPH, ce qui souligne l'importance de la β-protrusion dans cette interaction.

Mots-clés : neurofibromatose de type I, LIMK2, VCP, SUMOylation, K1731, signalisation cellulaire