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Deciphering the Molecular Mechanism of Pathogenicity in Destabilized variants of Neurofibromin mutated in its SecPH domain

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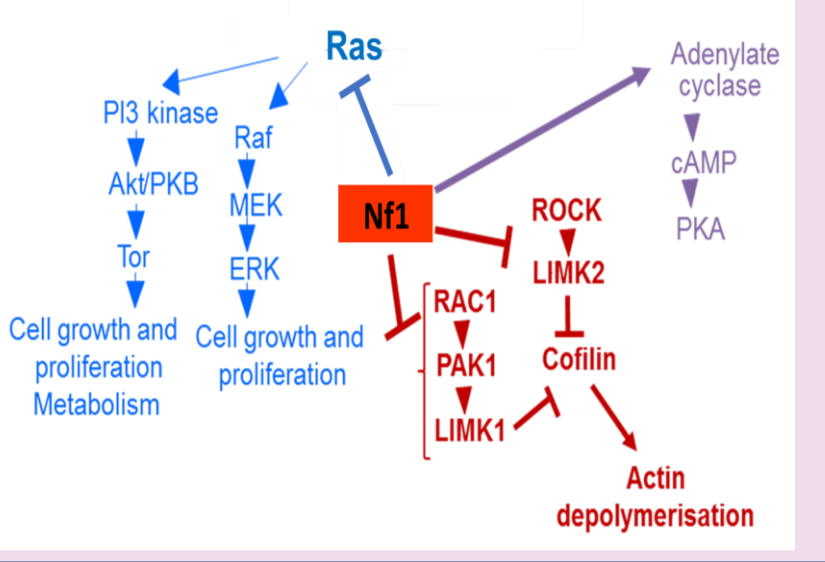
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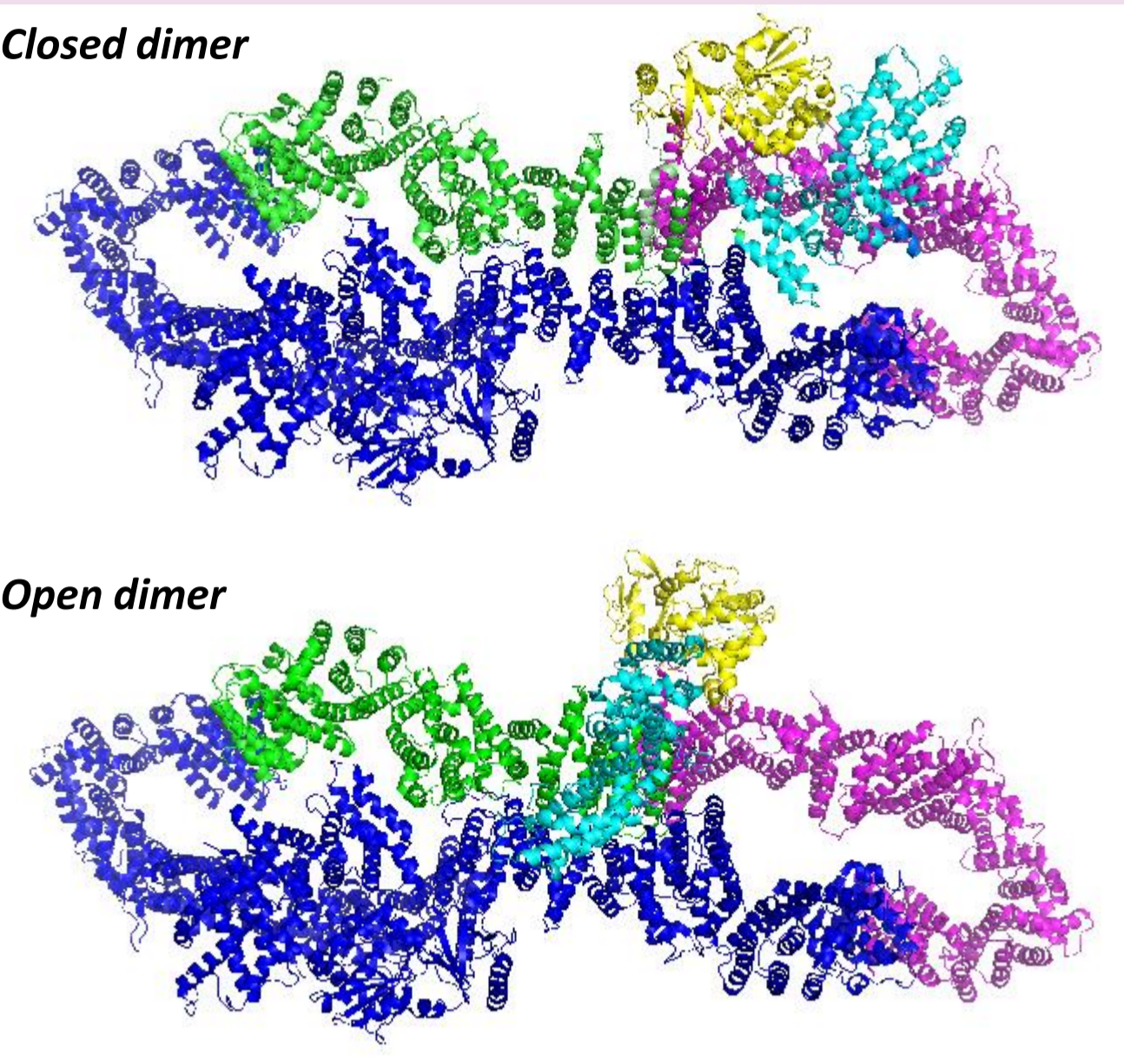
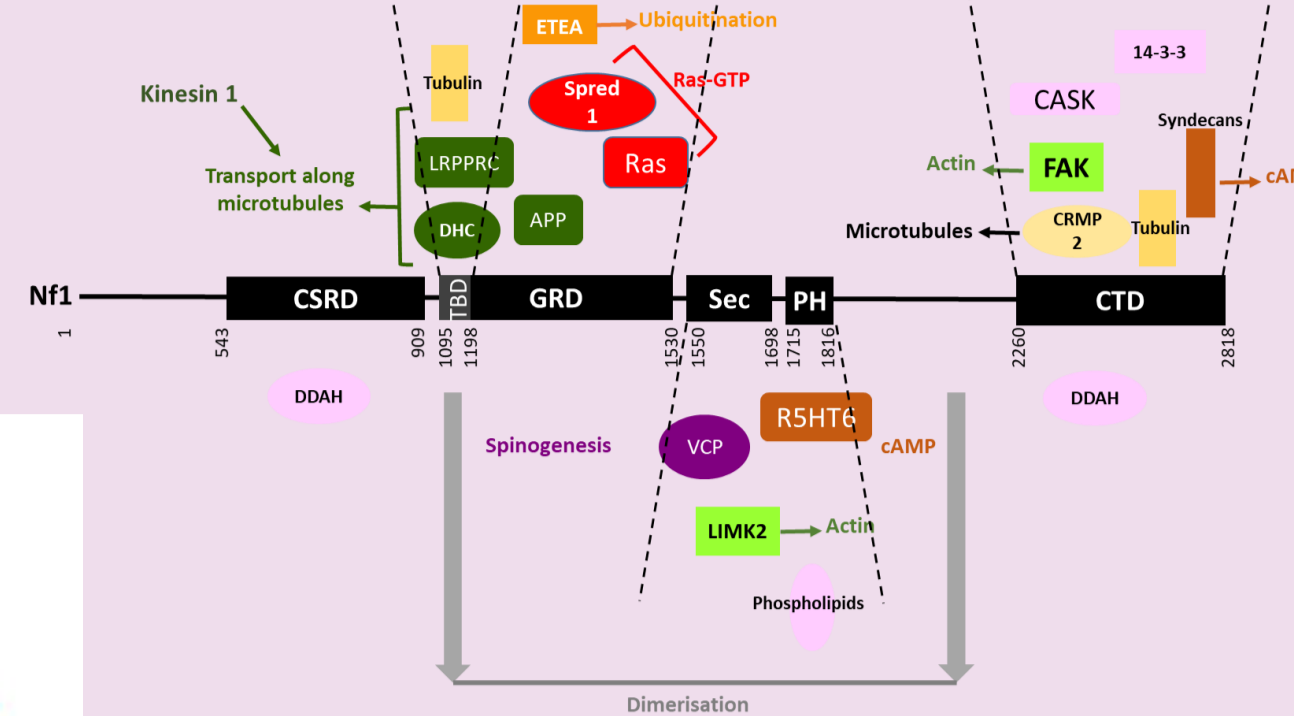
Introduction

1/ NF1 encodes Neurofibromin (Nf1) protein
-NF1 gene spans over 350kb and contains 60 exons.
-Alternative splicing: different isoforms produced
-Major isoform is isoform 2, encoding a 2,818 amino-acid Nf1
-Isoform 1 comprises 2839 aa and contains 21 additional aa in the GRD domain (30alt31 exon)

2/ Nf1 is multifunctional
-Nf1 has a Ras-GAP (GTPase activating protein) activity localized in its GRD (GAP related domain). It downregulates Ras-effector pathways (PI3K/mTOR, Raf/MEK/ERK)
-Nf1 positively regulates cAMP level
-Nf1 favors actin depolymerisation by inhibiting the ROCK/LIMK2/Cofilin and PAK1/LIMK1/Cofilin pathways.

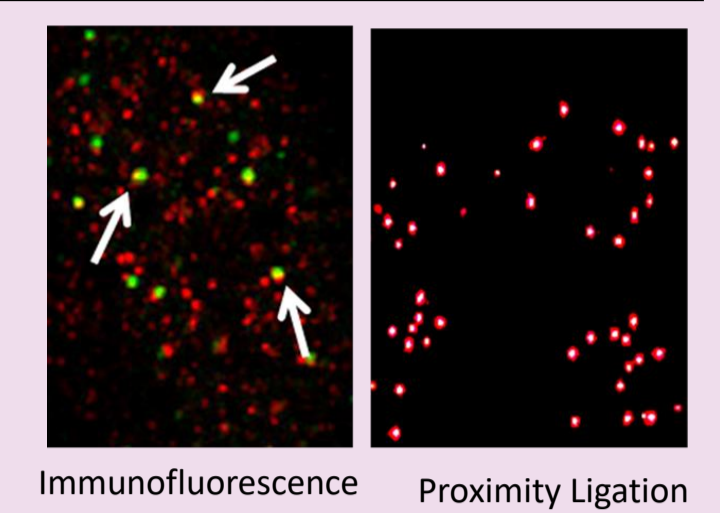
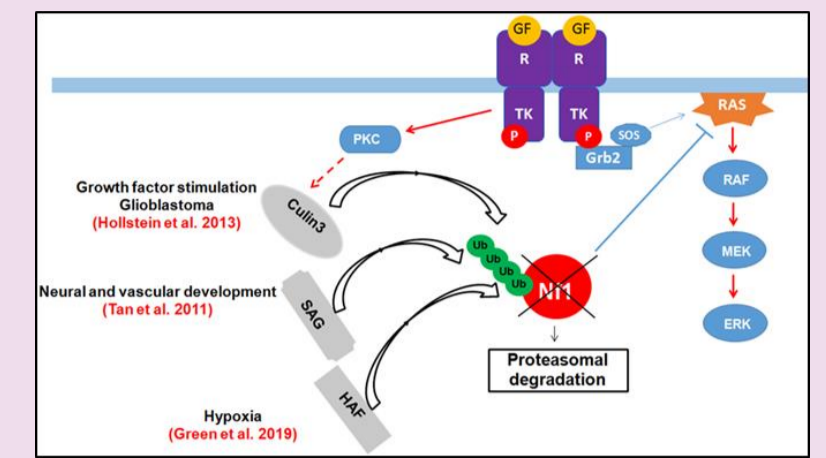


3/ Nf1 has many partners and domains
Nf1 interacts with many partners and is divided into different domains. GRD carries the Ras-GAP activity

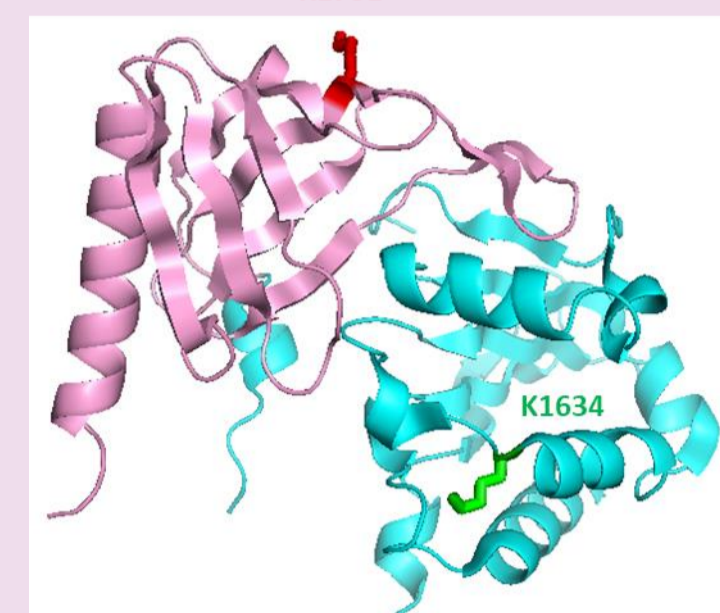
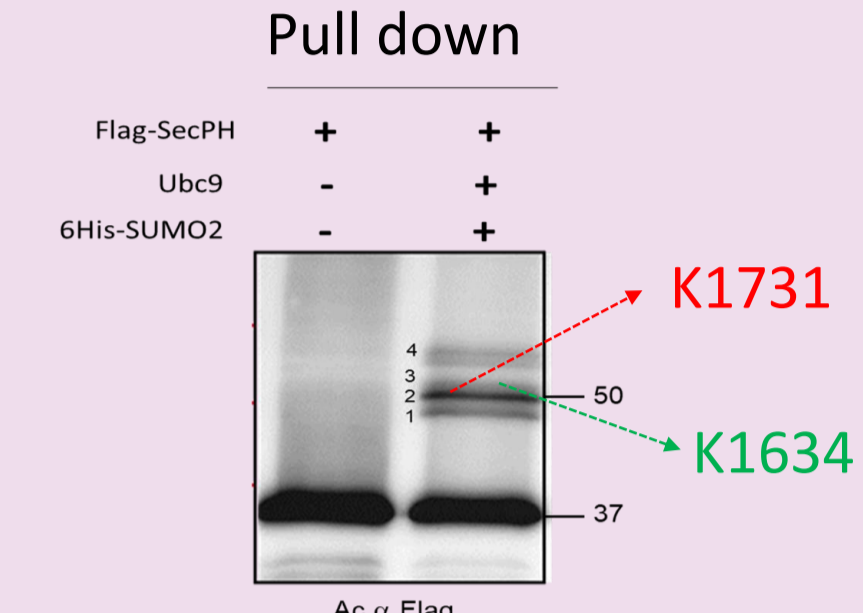


4/ Nf1 forms a dimer
Nf1 dimerises with two distinct conformations (Chaker-Margot, M., Werten, S., Dunzendorfer-Matt, T., Lechner, S., Ruepp, A., Scheffzek, K., Maier, T. (2022) Mol Cell 82: 1288)
-a closed auto-inhibited conformation with occluded Ras-binding site
-an asymmetric open and active conformation with an exposed Ras-binding site

5/ Nf1 is modified post-translationally
-Phosphorylation: CTD is phosphorylated by PKA (interaction with 14-3-3) and PKCe (nuclear entry).
-Ubiquitylation: different ubiquitin ligases ubiquitylate Nf1 and induce its proteasomal degradation.



-SUMOylation:
*Nf1 colocalises with PML (Promyelocytic Leukemia) nuclear bodies.
*Nf1 isoform 1 is SUMOylated on two lysines of the exon
*Nf1 isoform 2 and Nf1 SecPH domain are SUMOylated on K1634 and K1731 (See details in poster B. Vallée).



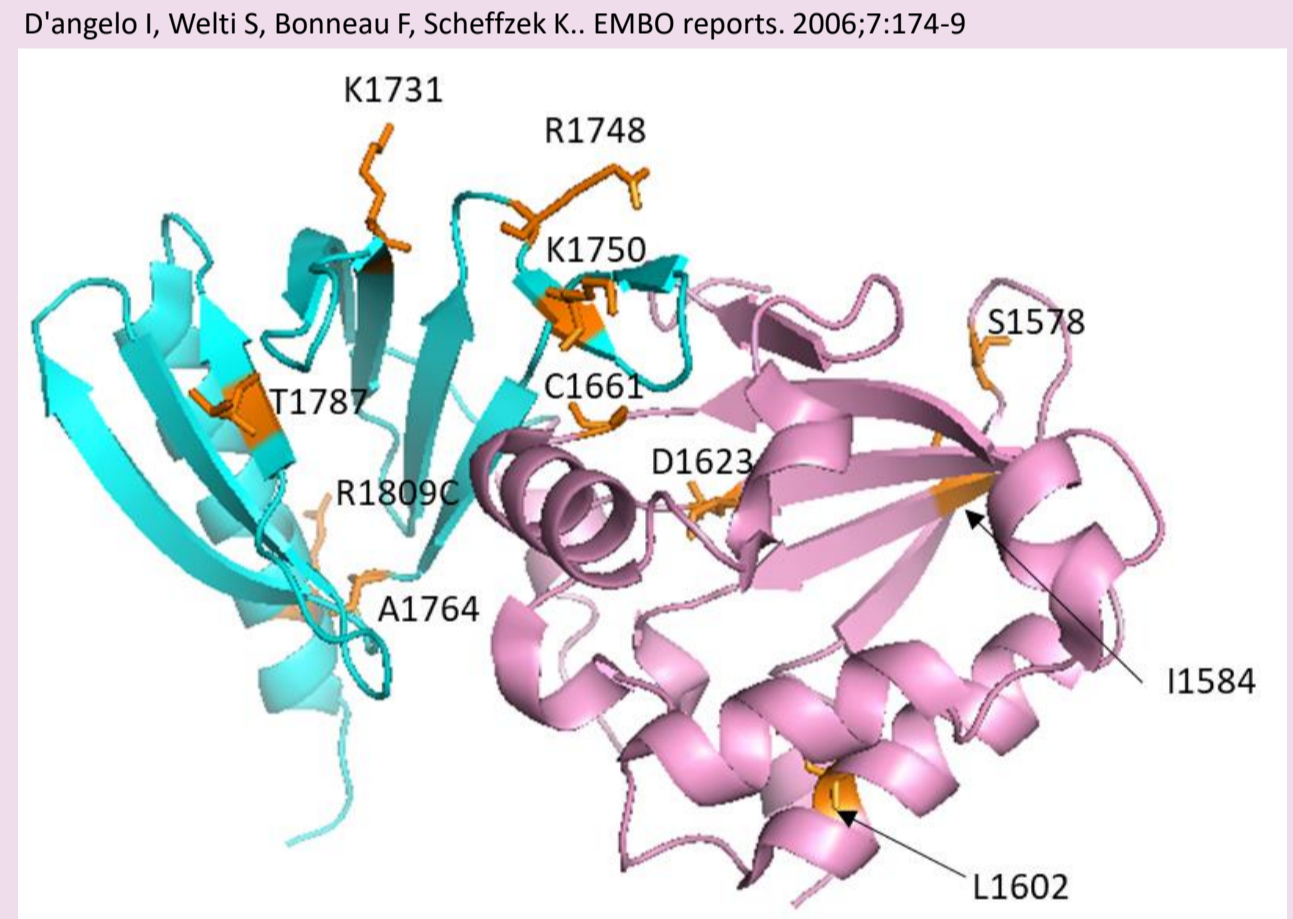
Goal of the study: Address the molecular basis responsible for the pathogenicity of different NF1 missense variants

Pathogenic mutants studied

1. Pathogenic mutations in Nf1
Almost 3000 different pathogenic mutations have been listed in NF1. They are distributed throughout the gene. They correspond to deletions, nonsense or missense mutations, these latter representing 17% of the total pathogenic variants.

2. Why studying pathogenic mutations in SecPH?
(i) SecPH is adjacent to GRD and might play an allosteric role on its activity, (ii) SecPH interacts with many proteins and phospholipids and might regulate Nf1 functions, (iii) SecPH is SUMO-modified and this might play a role in Nf1 functions, (iv) SecPH is exposed at the surface of the dimer and it is prone with its adjacent GRD domain, to a structural rearrangement highlighting its functional importance.

3. Mutants
Small deletion mutant: c.5205+5G>(p.Phe1719_Val1736del) (Sabbagh et al., 2013)
Missense mutants: c.4733C>T (p.Ser1578Phe) (Sabbagh et al., 2013)
c.4750A>G (p.Ile1584Val) (Bendova et al., 2007)
c.4805T>G (p.Leu1602Arg) (Pros et al., 2006)
c.4868A>G (p.Asp1623Gly) (Sabbagh et al., 2013)
c.4981T>C (p.Cys1661Arg) (Pasmant et al., 2015)
c.5290G>T (p.Ala1764Ser) (Han et al., 2001)
c.5360C>T (p.Thr1787Met) (Lee et al., 2006)
c.5425C>T (p.Arg1809Cys) (Pinna et al., 2015)
c.5192A>G (p.Lys1731Arg)
c.5306G>C (p.Arg1748Pro)
c.5306G>A (p.Arg1748Gln)
c.5311A>G (p.Lys1750Asp)



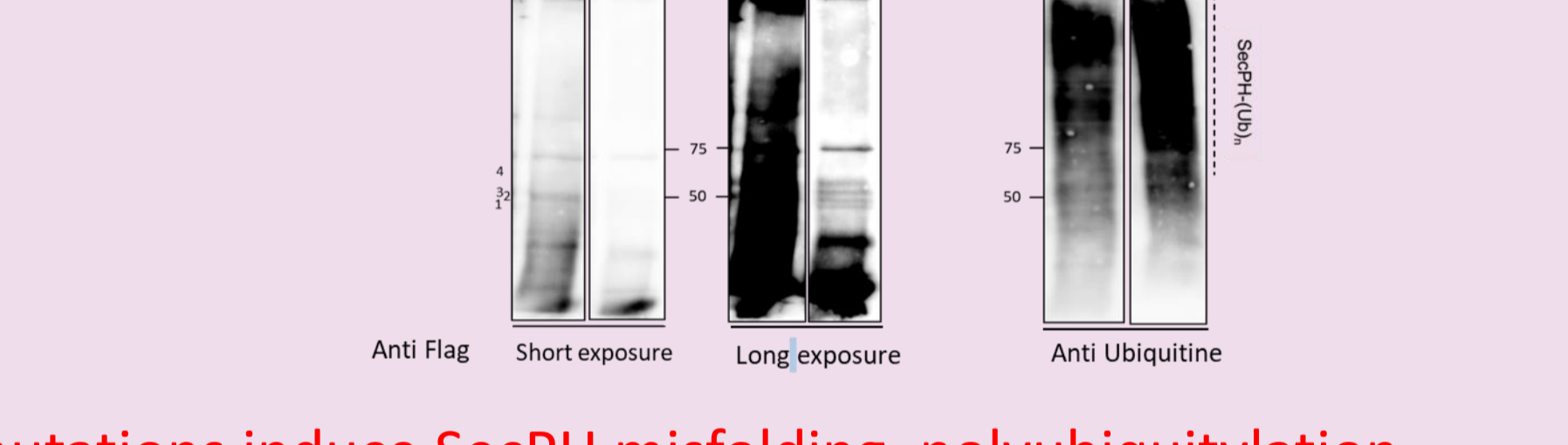
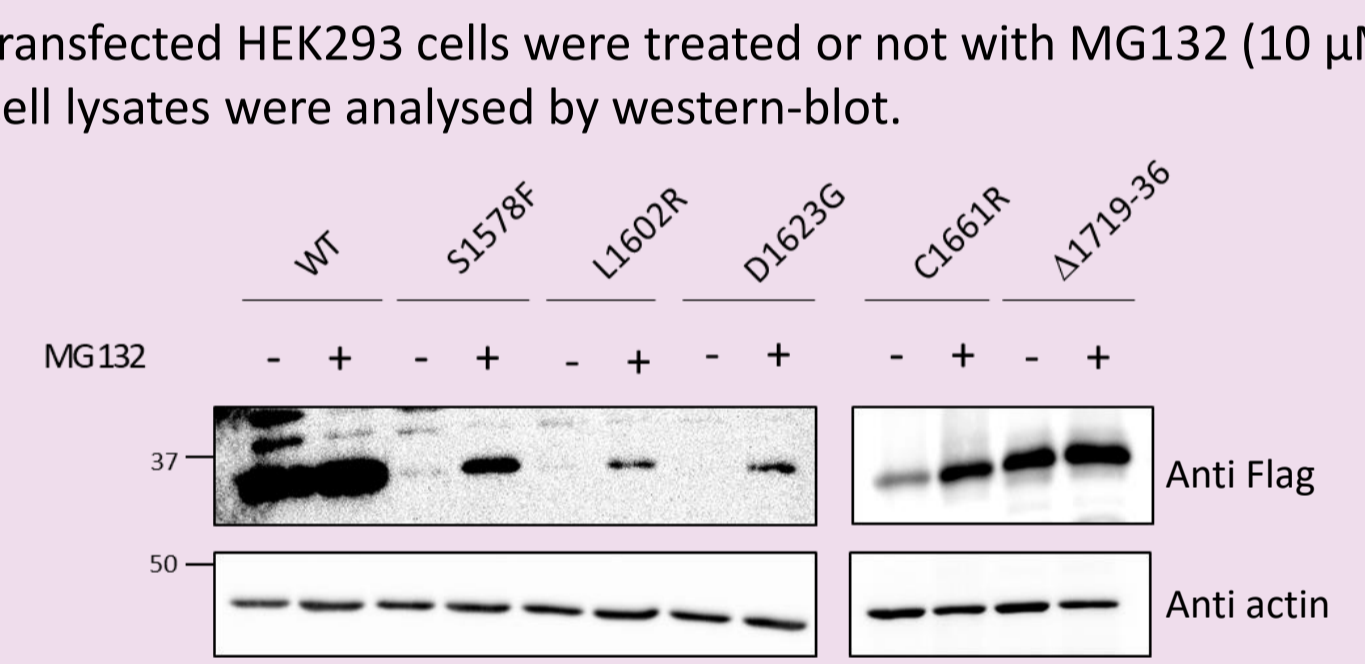
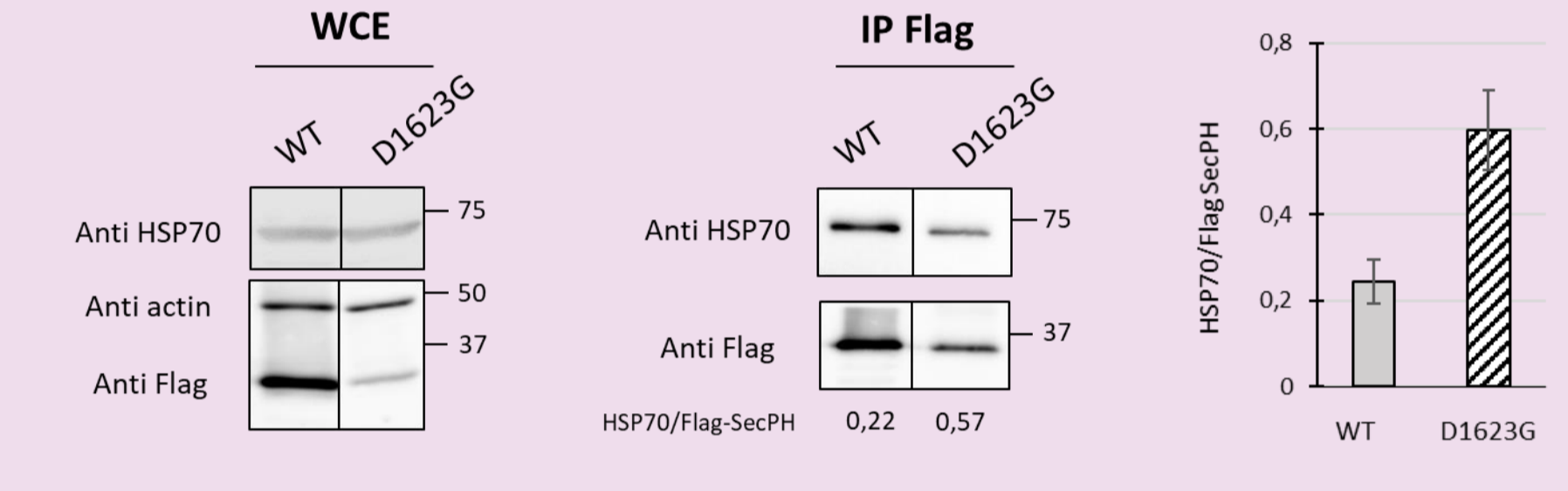
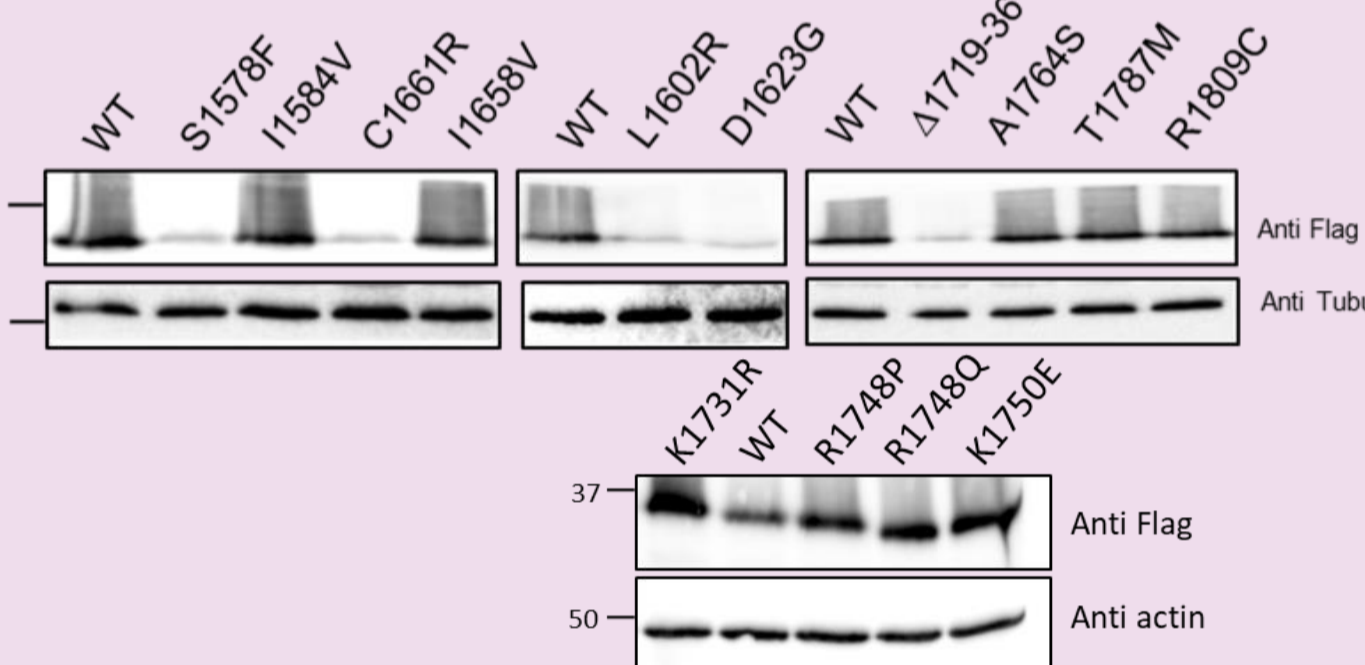
Biochemical study of SecPH mutants

1. SecPH mutants are not produced at the same basal level
Mutations were introduced in the p3XFlag plasmid encoding the Flag-SecPH. Resulting plasmids were transfected into HEK293 cells and cell lysates were analysed by western-blot.

2. Unstable SecPH mutants are stabilized by a proteasome inhibitor
Transfected HEK293 cells were treated or not with MG132 (10 μM). Cell lysates were analysed by western-blot.

3. Unstable SecPH mutants interact more strongly with the Hsp70 chaperone
Transfected HEK293 cells were treated with MG132. Cell lysates were immunoprecipitated with anti Flag beads and eluates were analysed by western blot.

4. Unstable SecPH mutants are polyubiquitylated
Same experiment as above but the IP Flag eluates were revealed with anti Flag and anti Ubiquitin antibodies



Structural data concerning SecPH mutants

Unstable SecPH mutants
These mutations include a large deletion (Δ1719-36) and missense mutations of buried residues into very different, typically larger, amino-acids (S1578F, C1661R, L1602R, D1623G), all of which may be expected to result in folding defects

Stable SecPH mutants
These mutations are either largely exposed on the surface (A1764S, T1787M, R1809C) or buried but then substituted with a similar hydrophobic residue (I1584V). Such mutations are unlikely to disturb the folding of the domain.

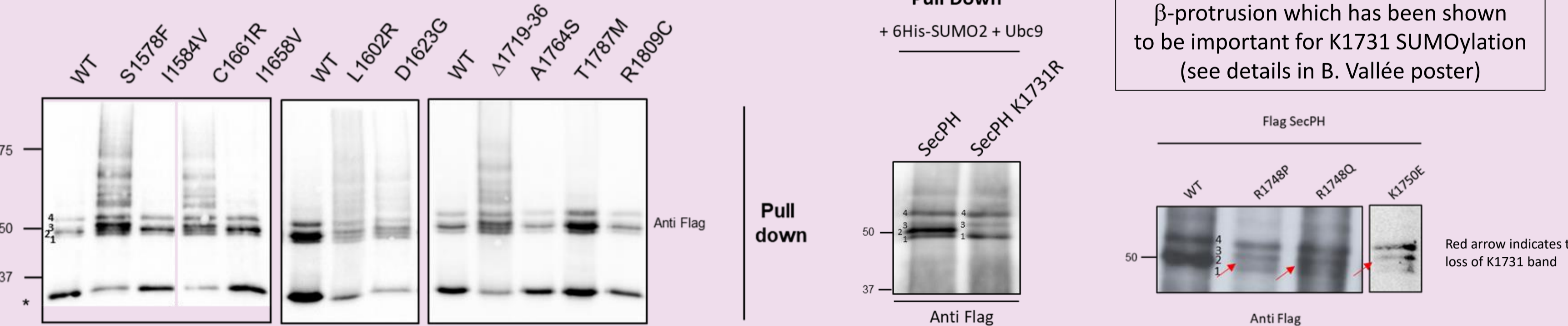


SUMOylation profile of SecPH mutants

HEK293 cells were co-transfected with pcDNA3 6His-SUMO2, pcDNA3 Ubq9, and plasmids encoding p3X Flag SecPH derivatives. After 24 h of growth, cell lysates were prepared in 6 M guanidinium buffer and SUMOylated proteins were enriched by pull down using cobalt beads. Pull down eluates were analysed by western-blotting and revealed with anti Flag antibodies.

These mutants are localized in the β-protrusion which has been shown to be important for K1731 SUMOylation (see details in B. Vallée poster)

SecPH mutants presented three types of profile: 1/ WT profile with 4 bands of SUMOylated SecPH, 2/ Profile without band 2 which corresponds to K1731 SUMOylation, 3/ PolySUMOylation profile of misfolded mutants



Transfer of mutations in full length Nf1 isoform2 tagged with Flag

Biochemical study

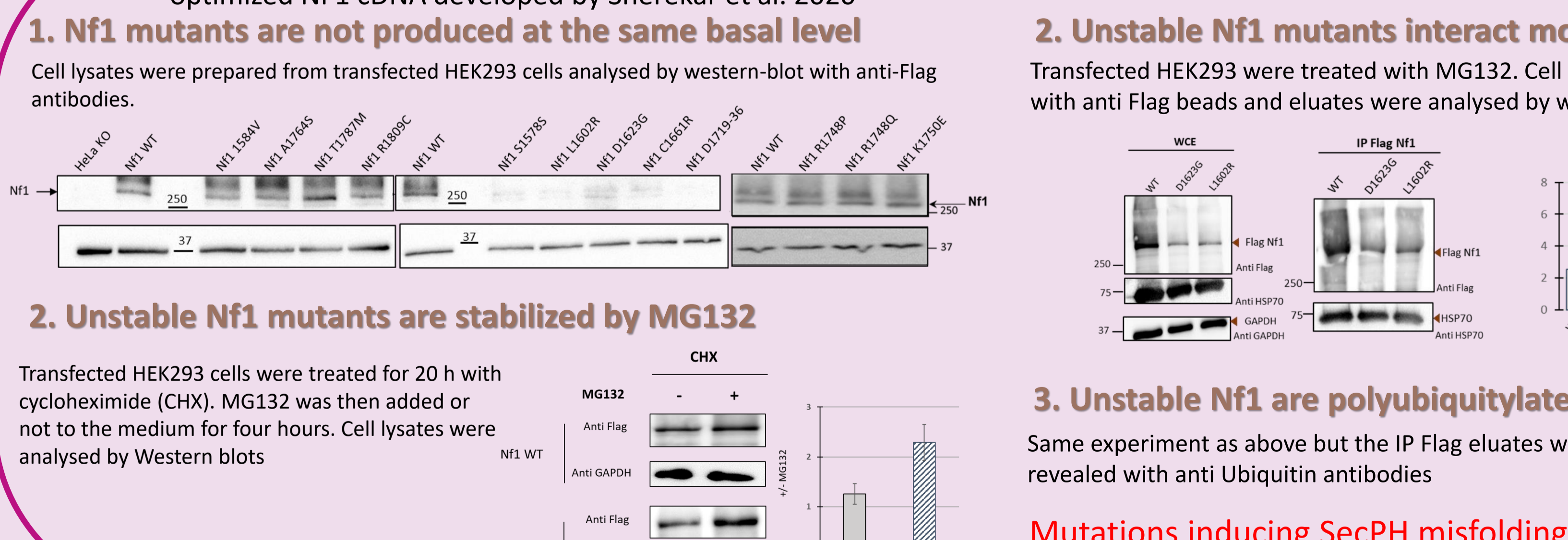
The plasmids encoding Flag-tagged Nf1 isoform 2 and its mutants were constructed based, in part, on a plasmid containing the codon optimized NF1 cDNA developed by Sherekar et al. 2020

1. Nf1 mutants are not produced at the same basal level
Cell lysates were prepared from transfected HEK293 cells analysed by western-blot with anti-Flag antibodies.

2. Unstable Nf1 mutants are stabilized by MG132
Transfected HEK293 cells were treated for 20 h with cycloheximide (CHX). MG132 was then added or not to the medium for four hours. Cell lysates were analysed by Western blots

3. Unstable Nf1 are polyubiquitylated
Same experiment as above but the IP Flag eluates were revealed with anti Ubiquitin antibodies

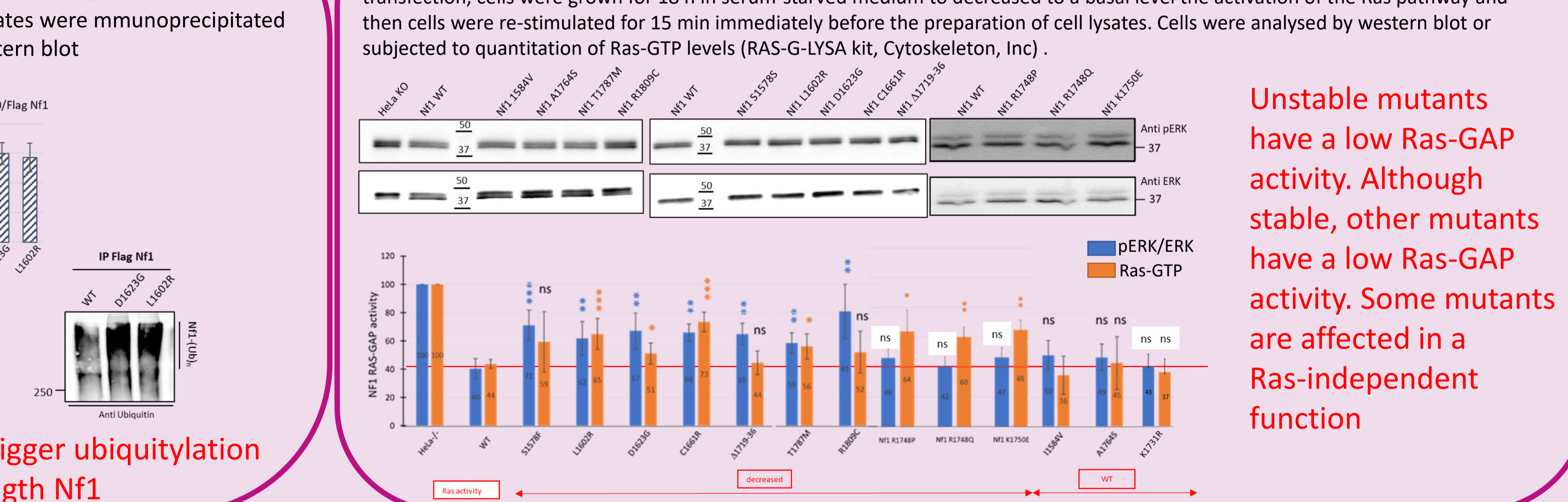
Mutations inducing SecPH misfolding trigger ubiquitylation and proteasomal degradation of full length Nf1



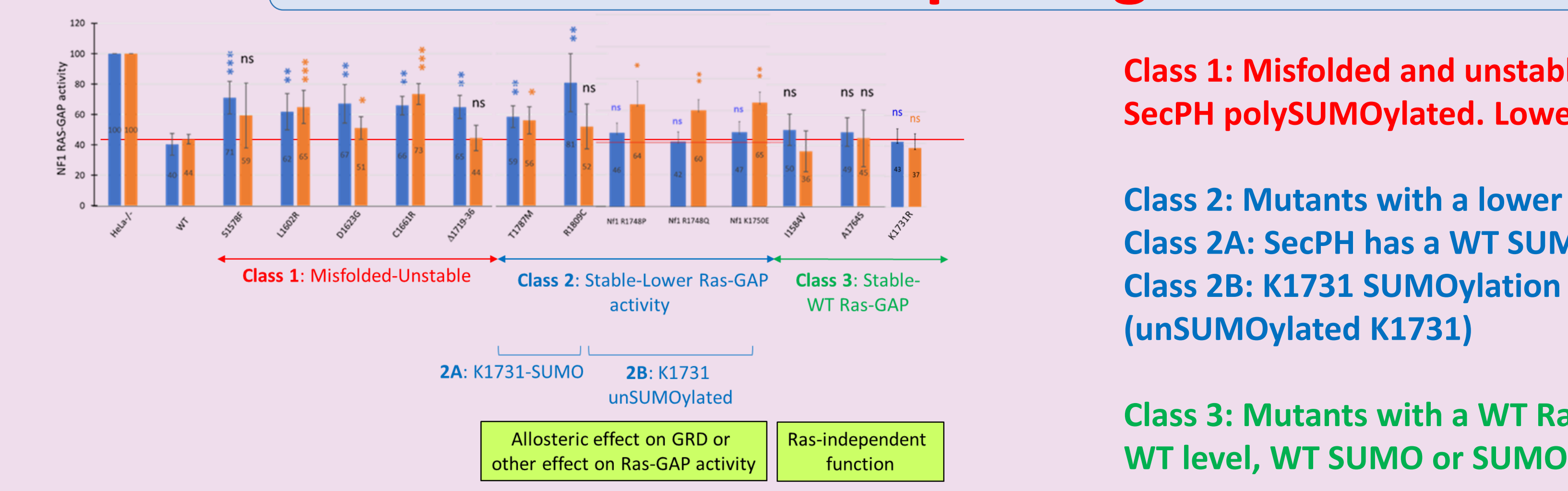
Ras-GAP activity of Nf1 mutants

HeLa KO (NF1-/-) cells were transfected with p3X Flag (empty vector), p3X Flag Nf1 WT or p3X Flag Nf1 derivatives. 24 h after transfection, cells were grown for 18 h in serum-starved medium to decrease to a basal level the activation of the Ras pathway and then cells were re-stimulated for 15 min immediately before the preparation of cell lysates. Cells were analysed by western blot or subjected to quantification of Ras-GTP levels (RAS-G-LYSA kit, Cytoskeleton, Inc).

Unstable mutants have a low Ras-GAP activity. Although stable, other mutants have a low Ras-GAP activity. Some mutants are affected in a Ras-independent function



Classification of the pathogenic mutants studied



Conclusion

1/ Some pathogenic mutations induce SecPH and full Nf1 misfolding resulting in polyubiquitylation and degradation by the proteasome
2/ Some pathogenic mutations have an allosteric effect on the GRD domain or affect GAP activity by another way.
3/ Some mutations are pathogenic because they affect a Ras-independent function of Nf1